PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/15, 15/81, 15/62
C07K 13/00, C12P 21/02
A61K 37/64, C12N 1/19
C12Q 1/37

A2

(11) International Publication Number: WO 93/09233

(43) International Publication Date: 13 May 1993 (13.05.93)

(21) International Application Number: PCT/US92/09400

(22) International Filing Date: 30 October 1992 (30.10.92)

(30) Priority data:
785,638 31 October 1991 (31.10.91) US

(60) Parent Application or Grant
(63) Related by Continuation
US 785,638 (CIP)
Filed on 31 October 1991 (31.10.91)

(71) Applicant *(for all designated States except US)*: THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES INC. [US/US]; 505 Coast Blvd. So., San Diego, CA 92186 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : WAGNER, Steven, L. [US/US]; 1295 Prospect Street, Unit D, La Jolla, CA 92037 (US). SIEGEL, Robert [US/US]; 4360 Huggins Street, San Diego, CA 92122 (US). THILL, Gregory, P. [US/US]; 75 Donald Street, Apartment 511, Weymouth, MA 02188 (US). HARPOLD, Michael, M. [US/US]; 1341 29th Street, San Diego, CA 92102 (US). COMER, William, T. [US/US]; 5460 Calzada Del Bosque, Rancho Santa Fe, CA 92067 (US).

(74) Agent: MISROCK, S., Leslie; Fennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

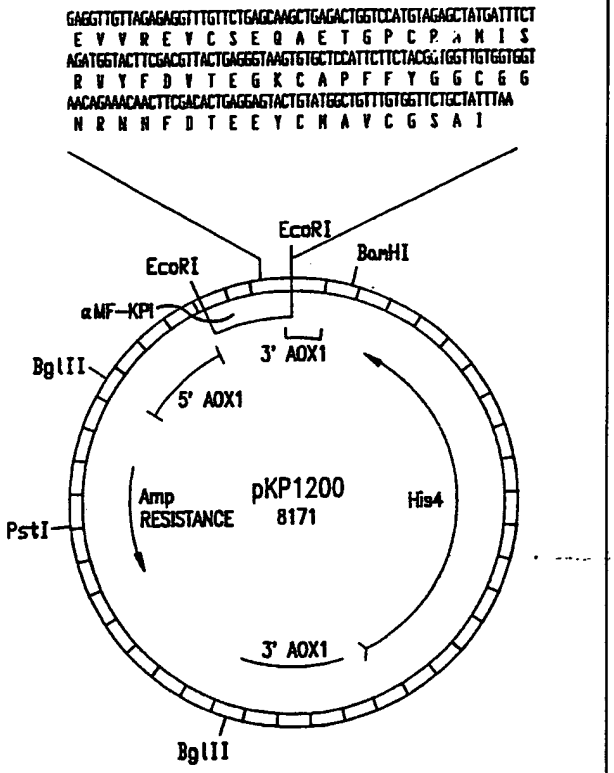
(81) Designated States: AU, BB, BG, BR, CA, CS, FI, HU, JP, KR, LK, MG, MN, MW, NO, PL, RO, RU, SD, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RECOMBINANT AMYLOID PRECURSOR PROTEIN INHIBITOR DOMAIN AND TREATMENT OF VARIOUS DISEASE STATES

(57) Abstract

The present invention relates to the production of a Kuniz type protease inhibitor (KPI) peptide, and its use in regulating protease activity associated with amyloidosis. This invention is further directed to *in vivo* and *in vitro* models of amyloidosis involving the KPI peptide. The KPI peptide can also be used to treat coagulation disorders by regulating proteases in the coagulation pathway. KPI is also used to inhibit or ameliorate protease-associated damage following injury, especially head injury. Nucleic acid sequences encoding the protease inhibitor and expression systems for high level expression of the active inhibitor are provided.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

RECOMBINANT AMYLOID PRECURSOR PROTEIN INHIBITOR DOMAIN AND TREATMENT OF VARIOUS DISEASE STATES

1. FIELD OF THE INVENTION

The present invention relates to the production of a Kunitz type protease inhibitor (KPI) peptide, and its use in regulating protease activity associated with amyloidosis. This invention is further directed to *in vivo* and *in vitro* models of amyloidosis involving the KPI peptide. The KPI peptide can also be used to treat coagulation disorders by regulating proteases in the coagulation pathway. KPI is also used to inhibit or ameliorate protease-associated damage following injury, especially head injury. Nucleic acid sequences encoding the protease inhibitor and expression systems for high level expression of the active inhibitor are provided.

2. BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) affects more than 30% of humans over 80 years of age, and as such, represents one of the most important health problems of developed countries (Evans et al., 1989, JAMA 262: 2551-2556; Katzman & Saitoh, 1991, FASEB J. 280:278-286). The etiology and pathogenesis of this progressive dementia is poorly understood, but symptomatic disease is associated histopathologically with amyloid plaques, neurofibrillary tangles and neuronal loss primarily in the temporal lobe and neocortex of the brain.

The amyloid β-protein is a 4.2 kDa peptide that is deposited in senile plaques and the cerebrovasculature of individuals afflicted with Alzheimer's disease and Down's syndrome (Glenner and Wong, 1984, Biochem. Biophys. Res. Commun. 120:885-890; Glenner and Wong, 1984, Biochem. Biophys. Res.

Commun. 122:1131-1135; Masters et al., 1985, EMBO J. 4:2757-2763; Masters et al, 1985, Proc. Nat'l. Acad. Sci. U.S.A. 82:4245-4249; Masters and Beyreuther, 1988, in Aging of the Brain, Volume 32, pp. 183-204, Raven Press, New York). It is derived from a larger precursor protein, the amyloid β-protein precursor (APP) (Goldgaber et al., 1987, Science 235:8778-8780; Kang et al., 1987, Nature 325:733-736; Robakis et al., 1987, Proc. Nat'l Acad. Sci. U.S.A. 84:4190-4194; and Tanzi et al., 1987, Science 235:880-884). Previous studies showed that APP can be translated from at least three alternatively spliced mRNAs to yield proteins of 695, ~~751~~ and 770 amino acids (Ponte et al., 1988, Nature 331:525-527; Tanzi et al., 1988, Nature 331:528-530; Kitaguchi et al., 1988, Nature 331:530-532). The latter two species contain an additional insert that encodes a domain that is homologous to Kunitz-type protease inhibitors (KPI) (Ponte et al., supra; Tanzi et al., supra; Kitaguchi, supra). The secreted form of APP protein which contains the KPI domain (KPI^+) was shown to be identical to the protease inhibitor protease nexin-2 (PN-2) (Van Nostrand et al., 1989; Nature 341:546-549; Oltersdorf et al., 1989, Nature 341:144-147). PN-2 is a potent inhibitor of certain serine proteases including factor XIa, trypsin, chymotrypsin, epidermal growth factor binding protein (EGF BP) and the γ-subunit of nerve growth factor (Van Nostrand et al., supra; Van Nostrand et al., 1990, J.Biol. Chem. 265:9591-9594; Smith et al., 1990, Science 248:1126-1128; Sinha et al., 1990, J.Biol.Chem. 265:8983-8985).

Previous studies have shown that KPI^+ APP mRNA is present, at varying levels, in most tissues, whereas KPI^- APP mRNA is found primarily in brain of normal individuals and AD patients (Ponte et al.,

supra; Tanzi et al., supra; Kitaguchi et al., supra; Golde et al., 1990, Neuron 4:253-267). Two major APP proteins with estimated molecular masses of 120 kDa and 105 kDa were found in human brain (Palmert et al., 1989, Proc. Nat'l. Acad. Sci. U.S.A. 86:6338-6342; Weidemann et al., 1989, Cell 57:115-126; Palmert et al., 1988, Biochem. Biophys. Res. Commun. 156:432-437). Immunoblot analysis with a polyclonal antiserum prepared against a synthetic peptide corresponding to a region of the KPI domain indicated that the 120 kDa form of APP protein is KPI^+ (Palmert et al., 1989, supra) and is thus probably PN-2. Lack of immunoreactivity with this same polyclonal antiserum suggested that the 105 kDa form of APP protein in brain is devoid of the KPI domain (Palmert et al., 1989, supra). These qualitative studies also suggested that the 105 kDa putative KPI^- form of APP is the predominant species of APP protein in brain, apparently consistent with the presence of abundant KPI^- APP mRNA in brain tissue.

Recent studies using protease inhibitory functional assays have shown that both the 105 kDa and 120 kDa APP proteins in normal and Alzheimer's disease brain contain the KPI domain (Van Nostrand et al., 1991, Proc. Nat'l Acad. Sci. U.S.A. 88:10302-10306). Moreover, KPI domain-specific precipitation assays reveal that KPI^- forms of APP protein represent less than 14% of total APP in brain (Van Nostrand et al., 1991, supra). Thus, although KPI^- APP mRNA is abundant in brain, little corresponding protein is present.

The proteolytic events that lead to the formation of the amyloid β-protein from APP remain unclear. Recent studies showed that release of the large extracellular domain of APP was the result of

proteolytic cleavage through the amyloid β-protein domain (Sisodia et al., 1990, Science 248:492-495; Esch et al., 1990, Science 248:1122-1124). This indicates that altered proteolytic processing events occur in Alzheimer's disease such that the integrity of the amyloid β-protein is maintained and it is ultimately liberated from APP.

2.1. ALTERED PROTEOLYTIC MECHANISMS MAY BE ASSOCIATED WITH AMYLOIDOSIS

Proteases and protease inhibitors have been recognized as potential contributors to the pathogenesis of Alzheimer's disease (Abraham, 1989, Neurobiol. Aging 10:463-5). However, various hypotheses have been developed concerning the role of proteolysis in Alzheimer's disease. Proteases metabolize proteins containing undesirable segments by "chopping" the segment, e.g., cleaving within the β-amyloid (A4) domain of APP. Proteases also play a detrimental role by processing proteins to release the pathogenic segment, e.g., cleaving the intact β-amyloid domain from APP. The question remains exactly how protease regulation affects β-amyloid deposition. As the following studies indicate, the matter remains unresolved.

In vitro proteolysis of APP at Lys 16 within the 42 amino acid β amyloid domain prevents amyloid formation and yields soluble APP (Esch et al., supra). Recent studies indicate slower APP processing and increased amounts of 16 kD pre-amyloid in early and late-onset familial Alzheimer's disease lymphoblastoid cell lines, suggesting that a deficiency in intra-amyloid β-protein proteolysis may play a role in amyloid deposition (Matsumoto and Fujiwara, 1991, Biochem. Biophys. Res. Commun. 175:361-365). In vitro work with transfected cells also showed that altered

APP processing results in release and subsequent deposition of amyloid-β protein, and that normal processing occurs within the amyloid β-protein region (Sisodia et al., 1990, Science 248: 1122-1124).

There is uncertainty regarding the role of KPI itself in amyloid processing, ranging from beneficial to detrimental, to inconclusive. Expression of an APP subdomain containing amyloid β-protein, but lacking the KPI sequence, resulted in cells that overproduced the amyloid β-protein epitope (Marotta et al., 1989, Proc. Nat'l. Acad. Sci. U.S.A 86:337-341). Increased production of mRNA for KPI-free APP has been suggested to contribute to the deposition of amyloid that occurs in Alzheimer's disease (Palmert et al., 1989, Prog. Clin. Biol. Res. 317:971-984). Another study suggested that inactivation of the KPI domain leads to amyloid deposition (DeFeudis, 1989, Neurobiol. Aging 10:467-468). However, other reports show that expression of mRNA encoding the APP-770 and 751 proteins (which include the KPI-domain) was elevated in Alzheimer's patients relative to normal controls (Tanaka et al., 1990, Rinsho Byori 38:489-493; Tanaka et al., 1989, Biochem. Biophys. Res. Commun. 165: 1406-1414). APP mRNA containing the KPI domain (APP-751, 770 and APP related 563) were increased relative to APP lacking KPI (APP-695) in the forebrain of aged rats with memory defects but not aged rats without behavioral impairments (Higgins et al., Proc. Nat'l. Acad. Sci. U.S.A. 87:3032-3036), supporting a model for detrimental effects of the amyloid precursor protein protease inhibitor. Other workers have also proposed that the APP KPI domain impairs the correct degradation of APP, resulting in amyloid deposition (Katanuma, 1990, Adv. Enzyme Regul. 30:377-392;

Johnson et al., 1988, Exp. Neurol. 102:264-268; Kitaguchi et al., 1988, Nature 331:530-532). Yet other reports suggest no direct role for the APP KPI domain based on relative mRNA expression in Alzheimer's (Koo et al., 1990, Neuron. 4:97-104; Spillantini et al., 1989, Brain Res. Mol. Brain Res. 6:143-150). Thus it is not clear what mechanisms are altered that result in improper processing of APP to amyloid β-protein.

Other protease inhibitors have been suggested to play a role in improper APP processing (Bauer et al., 1991, FEBS Lett 285:111-114). Addition of alpha 2-macroglobulin, a protease inhibitor, inhibited secretion of APP by cultured neuronal cells, suggesting a detrimental effect on proteolytic processing (Ganter et al., 1991, FEBS Lett 282:127-131). Alpha 1-antichymotrypsin has been identified in amyloid β-protein deposits in brains of patients with Alzheimer's disease, Down's syndrome, normal aging, HCHWA-Dutch type (Abraham et al., 1990, Neurobiol. Aging 11:123-129; Abraham and Potter, 1989, Prog. Clin. Biol. Res. 317:1037-1048; Abraham and Potter, 1989, Ann. Med. 21:77-81; Abraham et al., Cell 52: 487-501), as well as sporadic cerebral amyloid angiopathy (Picken et al., 1990, J. Neuropathol. Exp. Neurol. 49:4148), although no significant relationship between alpha 1-antichymotrypsin and alpha 1-trypsin levels in cerebrospinal fluid and Alzheimer's disease was observed (Delamarche et al., 1991, Neurobiol. Aging 12:71-74). Altered amyloid processing and the role of peptides or protease inhibitors have been implicated in neuronal ceroid lipofuscinosis (Wisniewski et al., 1990, Neurosci. Lett. 120:94-96; Wisniewski et al., 1990, Acta Neuropathol. 80:26-34) and cerebral amyloid angiopathy (Vinters et al., 1990,

Am. J. Pathol. 137: 233-240; Vinters et al., 1990, Ann. Neurol. 28:34-42). Although the exact pathological mechanisms are unknown, head injury has been associated with amyloidosis. For example, head trauma with loss of consciousness has been reported as increasing risk of AD (Chandra et al., 1989, Neurol. 39:1576-1578), and neurofibrillary tangles bearing β-amyloid antigens have been found in some cases of dementia pugilistica (Allsop et al., 1990, Am. J. Pathol. 136:255-260).

2.2. KUNITZ-TYPE PROTEASE INHIBITORS

The identification of a Kunitz-type protease inhibitor in one form of amyloid precursor protein (Ponte et al., 1988, Nature 331:525-527; Tanzi et al.,ibid., 528-530; Kitaguchi et al., ibid, 530-532) provided an unexpected occurrence of a protease inhibitor in a disease-associated protein, and suggested a role for protease activity in Alzheimer's disease (Carrell, ibid, 478-479).

Kunitz type protease inhibitors are a family of molecules such as aprotinin, also known as bovine basic pancreatic trypsin inhibitor, pancreatic Kunitz inhibitor, and trypsin-kallikrein inhibitor, a globular polypeptide of 58 amino acid residues. Aprotinin and aprotinin-type inhibitors have three disulfide bonds, resulting in an extremely stable molecule (Gebhard et al., 1986, in Proteinase Inhibitors, Barrett and Salvesen (eds), Elsevier Science Publishers BV, pp. 375-388). The reactive center sequences of many of these proteins are highly conserved, as shown in the following table (from Carrell, supra).

TABLE I.
Reactive Center Sequences

	P_2 P_1 P_1' P_2' P_3'	
APP	-Cys-Arg-Ala-Met-Ile	
Inter-α-TI(II)	-Cys-Arg-Ala-Phe-Ile	Kunitz
Aprotinin	-Cys-Lys-Ala-Arg-Ile	
P.Nexin	-Ala-Arg-Ser-Ser-Pro	
PAI-1	-Ala-Arg-Met-Ala-Pro	Serpin
PAI-2	-Gly-Arg-Thr-Gly-His	
Peptide	-Pro-Arg-CH_2Cl	Synthetic

The table shows the general homology of amyloid precursor protein inhibitor domain with the Kunitz inhibitors (inter-α-trypsin inhibitor domain II and aprotinin). The reactive center of each inhibitor acts as a putative substrate for its target enzyme which cleaves the P_1-P_1' bond. There is a common active site P_1 arginine in the Kunitz domain of the serpins (protease nexin 1 and plasminogen activator inhibitors PAI 1 and 2) and the synthetic peptide inhibitor shown in Table I. These similarities suggest overlapping inhibitory activity against serine proteases cleaving at arginyl residues.

The protease inhibitory activity of APP KPI has been well characterized in the native molecule from transfected cells (Kitaguchi et al., 1988, Nature 331:250-252; Gottfried and Octave, 1990, Biochem. Biophys. Res. Commun. 171:1015-1021) and purified from non-neuronal cells (Van Nostrand et al., 1990, J.Biol. Chem. 265:9591-9594; Smith et al., 1990, Science 248:1126-1128; Van Nostrand and Cunningham, 1987, J. Biol. Chem. 262:8508-8514; Knauer et al., 1983, J. Cell Physiol. 117:385-396; and Knauer and Cunningham, 1982, Proc. Nat'l. Acad. Sci. U.S.A. 79:2310-2314).

2.3. EXPRESSION OF AMYLOID PRECURSOR PROTEIN AND KPI FRAGMENTS OF APP

Kitaguchi et al. characterized APP containing KPI expressed in COS calls (Kitaguchi et al., 1988, supra). Two forms of APP, the 695-amino acid form and 751-amino acid form, have been expressed in a baculovirus system (Knops et al, 1991, J. Biol. Chem. 266:7285-7290). Expression of the 751 form of APP and fragments of APP, including amyloid β-protein and KPI as fusion proteins, has been described (International Publication Number WO 90/14841).

The 57-amino acid Kunitz domain of APP has been expressed in a fusion protein in bacteria (Sinha et al., 1990, J. Biol. Chem. 265:8983-8985). The fusion protein was shown to inhibit trypsin, epidermal growth factor-binding protein, α-chymotrypsin and the γ-subunit of nerve growth factor (Sinha et al., supra). Expression of a functional 57-amino acid KPI in E.coli has also been reported (Schilling et al., 1991, Gene 98:225-230). Bruns et al. (EP A1 0393431, published October 24, 1990) discloses homologs of the Alzheimer protease inhibitor.

Replacement of the P_1 Arg residue of a Kunitz-type protease inhibitor with other amino acids can alter the protease inhibitory properties of the inhibitor. For instance, replacement of the P_1 Arg of the APP KPI with a valine (Val) residue reportedly decreased the inhibitory potency of KPI against the trypsin-like proteases and chymotrypsin, while significantly increasing the inhibitory potency of KPI against neutrophil elastase (Sinha et al., 1991, J. Biol. Chem. 266:21011-21013).

3. SUMMARY OF THE INVENTION

The present invention addresses the need for a plentiful source of Kunitz-type protease inhibitor, in particular the Kunitiz-type protease inhibitor of amyloid precursor protein, by providing for high level expression of the KPI domain or an analog thereof. Specialized DNA vectors which provide for high level expression in yeast are described.

The present invention solves the need for plentiful source of KPI lacking exogenous portions of APP, especially the A4 region, for use in *in vivo* and *in vitro* models for amyloidosis, and, in the appropriate dosage range, for treating or preventing amyloidosis, since it is believed that altered proteolytic processing is involved in amyloidosis. Thus the invention provides for administering an appropriate dosage of the Kunitz-type protease inhibitor for treatment and/or prevention of a disease characterized by amyloidosis, such as Alzheimer's disease, Down's syndrome, neuronal ceroid lipofuscinosis, cerebral amyloid angiopathy, and hereditary cerebral hemorrhage with amyloidosis - Dutch and Icelandic, as well as amyloidosis associated with traumatic head injury and *demantia pugilistica*. Moreover, it is a further advantage that the protease inhibitors of the invention (KPI and analogs thereof) are generally useful for treating certain conditions involving proteases, e.g., coagulation disorders. In a specific example, *infra*, KPI inhibits Factor XIa in a heparin-independent fashion. In another embodiment, KPI inhibits proteases associated with brain damage following head trauma. In yet another aspect, a protease inhibitor of the invention inhibits elastase enzymes that may be involved in pathological conditions such as cystic fibrosis, acute

pancreatitis, and emphysema. Another KPI of the invention is a more potent chymotrypsin inhibitor than the KPI of the amyloid precursor protein. The protease inhibitor may be the Kunitz domain of amyloid precursor protein, or it may be another Kunitz-type protease inhibitor that has been modified to have the same protease specificity as the Kunitz domain of amyloid precursor protein, or an analog of the KPI of amyloid precursor protein with altered specificity.

3.1. ABBREVIATIONS

AD	Alzheimer's Disease
APP	Amyloid precursor protein
APP-ase	Putative protease specific for APP that releases β-amyloid
A4	β-Amyloid peptide
HCHWA	Hereditary cerebral hemorrhage with amyloidosis
KPI	Kunitz-type protease inhibitor
αMF	alpha(α) mating factor

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure. 1. A) Diagram of expression plasmid pAO815. B) Diagram of recombinant expression plasmid pKPI200 (see SEQ ID NO. 1). Both diagrams are to scale.

Figure. 2. Inhibition of trypsin activity by KPI (closed circles, 1 mg/ml) and broth (closed triangles). Aprotinin (open squares, 1 mg/ml) served as a positive control.

Figure 3. Stoichiometric inhibition of trypsin activity by KPI. A solution of 10 nM trypsin was titrated with purified KPI and the remaining

protease activity measured. The titration curve shows that a 1:1 ratio of KPI to trypsin substantially inhibited trypsin activity when both molecules are present at a 10 nM concentration.

Figure 4. Influence of KPI on the secretion of APP by SHSY5Y human neuroblastoma cells. The cells were incubated for the indicated periods with KPI, or with PN-2, and the secretion of APP detected on Western blots of concentrated conditioned media using the anti-PN-2 monoclonal antibody mAbP2-1 as the probe (see Section 9, infra). A) Conditioned media of cells exposed to 1 μM KPI. Note that at this concentration of KPI does not cause any band shift of the APP. B) Conditioned media of cells exposed to 10 μM KPI. Note the presence of two forms of APP. C) Conditioned media of cells exposed to 100 μM KPI. Note that all or nearly all of the APP is found in the truncated form. The APP bandshift represents a C-terminal truncation because the truncated form is recognized by mAbP2-1, which is specific for the N-terminus of APP.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention involves high level expression of KPI in yeast, and the use of KPI expression products in the treatment of various diseases and disorders. In one embodiment, high level expression of a synthetic KPI gene provides sufficient product for treatment of patients in need of such therapy.

The present invention is based, in part, on the production of large quantities of the KPI, e.g., the Kunitz-type protease inhibitor domain of amyloid precursor protein (APP), and the discovery that KPI so produced may be used in the development of a therapeutic treatment and prevention of diseases

associated with amyloidosis, including but not limited to Alzheimer's disease, Down's syndrome, neuronal acid lipofuscinosis, cerebral amyloid angiopathy, or hereditary cerebral hemorrhage with amyloidosis (HCHWA) - Dutch type and Icelandic type, as well as amyloidosis associated with head injury and dementia pugilistica. In one embodiment, an appropriate dose of the KPI peptide can be used in a therapeutic treatment or prevention of a disease associated with amyloidosis. The KPI peptide can also be used to find other factors that are crucial in processing of APP, such as the proteases involved in normal and amyloidogenic processing of APP. The protease responsible for processing of APP that does not generate amyloid is most likely a serine protease that cleaves after lysine-16 of the β-protein domain of APP when inserted in the plasma membrane of a cell. The product of this cleavage of APP (containing a KPI domain) is protease nexin-2 (PN-2).

In another embodiment, high level expression of the synthetic KPI gene provides sufficient amounts of KPI for therapeutic treatment of coagulation disorders and inhibition of proteolysis elicited by head injury. In still another embodiment, high level expression of a KPI analog with altered specificity provides therapeutic amounts of KPI for treatment of pancreatitis, emphysema, cystic fibrosis and other pathological conditions involving proteolytic activity.

5.1. PRODUCTION OF KPI

KPI for use in the present invention can be produced by any method, including but not limited to chemical synthesis, proteolytic cleavage from APP, or

expression of a gene, such as a cDNA, in a recombinant microorganism.

In a preferred embodiment, the KPI is expressed in yeast; more preferably, the KPI is expressed in a methylotrophic yeast. Expression of KPI in methylotrophic yeast provides many advantages over other methods. For example, formation of the correct disulfide linkages is very difficult in chemical synthesis when more than one pair of cysteines are present in a sequence. Proteolytic cleavage of KPI from APP requires specific proteolysis at amino acid residues adjacent to but not within the KPI domain. Identification of such protease targets, determining correct proteolysis conditions, and isolating the products all require substantial effort. Expression of KPI from recombinant bacteria or mammalian cells only provides a few mg of product per ml of culture.

In contrast, as demonstrated in the Examples, infra, expression of KPI in methylotrophic yeast provides large quantities (almost gram/l) of product with the structure necessary for activity. Preferably, expression of KPI in yeast provides yields of about 0.1 g/l; more preferably about 1 g/l.

The nucleotide sequence encoding KPI can be a cDNA or a synthetic gene. As used herein, the term "synthetic KPI gene" refers to a nucleic acid sequence that incorporates codons utilized preferentially in high level expression of a yeast gene. In a specific embodiment, a synthetic gene is formulated by back translation of the amino acids of the KPI of amyloid precursor protein (amino acids 285-345 of APP; SEQ ID NO. 2), or an analog thereof, with codons selected based on preferential utilization in highly expressed P. pastoris genes. For this purpose, a codon

frequency program, such as that prepared by the University of Wisconsin Genetics Group, can be used in combination with consensus data generated from known gene sequences of the yeast to formulate a sequence.

Any method known in the art may be used to prepare a gene encoding KPI for high level expression in yeast, more preferably in methylotrophic yeast, including chemical synthesis and site-directed mutagenesis of a cDNA encoding a KPI. In a specific embodiment, overlapping oligonucleotides encoding the amino acid sequence are synthesized on an ABI 380A DNA synthesizer using phosphoramidate chemistry. Preferably the synthetic gene is synthesized to include endonuclease sites at the 5' end and the 3' end to facilitate the construction of recombinant vectors used to clone the synthetic gene. For example, in a specific embodiment, the synthetic gene is synthesized with a HindIII site at its 5' end and both an EcoRI and a BamHI site at its 3' end. Preferably oligonucleotides are purified, then annealed to form the synthetic gene. The desired product, based on size, is isolated using standard procedures, and inserted in a cloning or expression vector.

Prior to insertion in an expression vector, the synthetic gene can be sequenced. Incorrect base deletions or insertions can be corrected, e.g., by site directed mutagenesis (Zolten and Smith, 1983, Meth. Enzymol 100:468). Furthermore, undesired sequences can be deleted, for example by site directed mutagenesis. The correct synthetic gene containing any additional regulatory sequences, including a secretion signal sequence, is then inserted in a yeast expression vector. Yeast cells, preferably methylotrophic yeast cells, and more preferably P.

pastoris, are transformed with the expression vector, preferably by the whole-cell lithium chloride yeast transformation procedure (Ito et al., 1984, Agric. Biol. Chem. 48:341), with modification necessary for adaptation to methylotrophic yeast, such as P. pastoris (see Cregg et al., U.S. Patent No. 4,929,555), or other known methods such as the spheroplast technique described by Cregg et al., 1985, in Mol. Cell Biol. 5:3376 and U.S. Patent No. 4,879,231.

The present invention, provides for high level expression of KPI from any recombinant yeast species. Preferably, the yeast species contemplated for use in the practice of the present invention are methylotrophs, i.e., species which are able to grow on methanol (as well as other) carbon source nutrient. Species which have the biochemical pathways necessary for methanol utilization fall into four genera, i.e., Candida, Hansenula, Pichia, and Torulopsis. Of these, a substantial amount is known about the molecular biology of members of the species Hansenula polymorpha and Pichia pastoris.

With respect to methylotrophic yeasts, it is known that the methylotrophic yeasts share a common biochemical pathway for methanol utilization employing the same enzymes (see, e.g., Craig et al., 1988, Dev. Ind. Microbiol. 29:33-41). Methylotrophic yeasts were classified as members of four genera, as described above, based on methanol utilization, DNA base composition, quinone systems, composition of cell wall mannans and the immunological relateness of the alcohol oxidases (see, e.g., EP A2 0173378; Lee et al., 1980, J. Gen. Appl. Microbiol. 26:133-158; Komagata, 1991, in Biology of Methylotrophs, Goldberg et al., eds. Butterworth-Heinemann, Chapter 2:

Systematics of Methylotrophic Yeasts). More recent classification systems have transferred all Torulopsis species that utilize methanol into the genus Candida (see, e.g., EP A2 0173378; Yarrow et al, 1978, Int. J. Syst. Bacteriol. 28:611-615) and all methanol-utilizing Hansenula species into the genus Pichia (see Kurtzman, supra). Thus it is recognized by the art that methylotrophic yeast species are closely related, share regulatory mechanisms, and can be used interchangeably without undue experimentation for the high level expression of KPI.

The presently preferred yeast species for use in the practice of the present invention is Pichia pastoris, a known industrial yeast strain that is capable of efficiently utilizing methanol as the sole carbon and energy source.

There are a number of methanol responsive genes in methylotrophic yeast, the expression of each being controlled by methanol responsive regulatory regions (also referred to as promoters). Any of such methanol responsive promoters are suitable for use in the practice of the present invention. Examples of specific regulatory regions include the promoter for the primary alcohol oxidase gene from Pichia pastoris AOX1, the promoter for the secondary alcohol oxidase gene from P. pastoris, AXO2, the promoter for the dihydroxyacetone synthase gene from P. pastoris (DAS), the promoter for the P40 gene from P. pastoris, the promoter for the catalase gene from P. pastoris, and the like.

The presently preferred promoter region employed to drive KPI synthetic gene expression is derived from a methanol-regulated alcohol oxidase gene of P. pastoris. P. pastoris is known to contain two functional alcohol oxidase genes: alcohol oxidase I

(AOX1) and alcohol oxidase II (AOX2) genes. The coding portions of the two AOX genes are closely homologous at both the DNA and the predicted amino acid sequence levels and share common restriction sites. The proteins expressed from the two genes have similar enzymatic properties but the promoter of the AOX1 gene is more efficient and thus the AOX1 gene is more highly expressed; therefore, its use is preferred for KPI expression. The AOX1 gene, including its promoter, has been isolated and thoroughly characterized. See Ellis et al., 1985, Mol. Cell. Biol. 5:1111 and U.S. Patent No. 4,855,231.

The expression cassette used for transforming methylotrophic yeast cells contains, in addition to a methanol responsive promoter of a methylotrophic yeast gene and the KPI encoding DNA sequence (KPI synthetic gene), a signal or leader sequence. The term signal or leader sequence refers to a sequence of amino acids that effect transport of a linked polypeptide through the cell membrane to release the mature KPI peptide into the cell culture medium. In particular, a signal sequence refers to a sequence of hydrophobic amino acids at the amino terminus of the protein to which it is linked. In addition, the signal sequence includes one or more sequences of amino acids that are recognized by one or more host cell proteases, called processing sites, interposed between the signal sequence and the protein, whereby removal of the signal or leader sequence may be effected.

The signal sequences contemplated for use in secreting KPI from methylotrophic yeasts are those that effect transport of KPI through the cellular membrane of the methylotrophic yeast. Any signal sequence and processing sites that are effective for

secreting mature KPI into the extracellular space of a methylotrophic host known to those of skill in the art may be used. Such signal sequences include but are not limited to the Saccharomyces cerevisiae alpha mating factor prepro signal (including the processing site lys-arg-$(glu-ala)_x$, wherein x is an integer between 0 and 3), the acid phosphatase pre-pro signal sequence, the invertase secretion signal sequence, human serum albumin signal sequence, the insulin pre-pro signal sequence, the lysozyme signal sequence, and the like. Preferably, the signal sequence will provide for secretion of relatively high concentrations (such as greater than 10 mg/l) of mature KPI. In a preferred embodiment, a DNA sequence encoding the in-reading frame S. cerevisiae alpha-mating factor (αMF) pre-pro sequence, including a DNA sequence encoding the processing site: lys-arg (also referred to as the lys-arg encoding sequence), and a transcription terminator functional in a methylotrophic yeast, is used.

The S. cerevisiae αMF is a 13-residue peptide, secreted by cells of the "alpha" mating type, that acts on cells of the opposite "a" mating type to promote efficient conjugation between the two cell types and thereby formation of "a-alpha" diploid cells (Thorner et al., 1981, The Molecular Biology of the Yeast Saccharomyces, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 143). The αMF pre-pro sequence is a leader sequence contained in the αMF precursor molecule, and includes the lys-arg encoding sequence which is necessary for proteolytic processing and secretion (see, e.g., Brake et al., 1984, Proc. Nat'l. Acad. Sci. USA, 81:4642).

The transcription terminator functional in a methylotrophic yeast used in accordance with the

present invention has either (a) a subsegment which encodes a polyadenylation signal and polyadenylation site in the transcript, and/or (b) a subsegment which provides a transcription termination signal for transcription from the promoter used in the expression cassette. The term "expression cassette" as used herein, and throughout the specification and claims, refers to a DNA sequence which includes sequences functional for both the expression and the secretion processes. The entire transcription terminator is taken from a protein-encoding gene, which may be the same or different from the gene which is the source of the promoter.

For the practice of the present invention it is preferred that multiple copies of the above-described expression cassettes be contained on one DNA fragment, preferably in the head-to-tail orientation.

The DNA fragments according to the invention optionally further comprise a selectable marker gene. For this purpose, any selectable marker gene functional in methylotrophic yeast may be employed, i.e., any gene which confers a phenotype upon methylotrophic yeast cells, thereby allowing them to be identified and selectively grown from among a vast majority of untransformed cells. Suitable selectable marker genes include, for example, selectable marker systems composed of an auxotrophic mutant P. pastoris host strain and a wild type biosynthetic gene which compliments the host's defect. For transformation of His4⁻ P. pastoris strains, for example, the S. cerevisiae His4 gene or P. pastoris gene, or for transformation of Arg4⁻ mutants, the S. cerevisiae ARG4 gene or the P. pastoris ARG4 gene, may be employed.

In addition, DNA fragments according to the invention optionally further comprise selectable

marker genes which are functional in bacteria. Thus, any gene can be used which confers a phenotype on bacteria that allows transformed bacterial cells to be identified and selectively grown from among a vast majority of untransformed cell. This additional selectable marker enables DNA of the invention to be transformed into bacteria such as E. coli for amplification. Suitable selectable marker genes include the ampicillin resistance gene (Amp^r), tetracycline resistance gene (Tc^r), and the like.

When it is contemplated to pass DNA of the invention through bacterial cells, it is desirable to include in the DNA construct a bacterial origin of replication, to ensure the maintenance of the bacteria. Exemplary bacterial origins of replication include the f1-ori, colisin, col E1, and the like.

If the yeast host is transformed with a linear DNA fragment containing the KPI synthetic gene under the regulation of a promoter region of a P. pastoris gene and αMF sequences necessary for processing and secretion, the expression cassette is integrated into the host genome by any of the gene replacement techniques known in the art, such as by one-step replacement (see, e.g., Rothstein, 1983, in Methods Enzymol. 101:202; Cregg et al., 1987, in Bio/Technology 5:479; and U.S. Patent No. 4,882,279) or by two-step gene replacement methods (see e.g., Scherer and Davis, 1979, in Proc. Natl. Acad. Sci. USA 76:4951). The linear DNA fragment is directed to the desired locus, i.e., to the target gene to be disrupted, by means of flanking DNA sequences having sufficient homology with the target gene to effect integration of the DNA fragment therein. One-step gene disruptions are usually successful if the DNA to be introduced has as little as 0.2 kb homology with

the fragment locus of the target gene; it is however, preferable to maximize the degree of homology for efficiency.

If the DNA fragment according to the invention is contained within, or is, an expression vector, e.g., a circular plasmid, one or more copies of the plasmid can be integrated at the same at different loci, by addition to the genome instead of by gene disruption. Linearization of the plasmid by means of a suitable restriction endonuclease facilitates integration.

The term "expression vector", as employed herein, is intended to include vectors capable of expressing DNA sequences contained therein, where such sequences are in operational association with other sequences capable of effecting their expression, i.e., promoter sequences. In general, expression vectors usually used in recombinant DNA technology are often in the form of "plasmids", i.e., circular, double-stranded DNA loops, which in their vector form are not bound to the chromosome. In the present specification the terms "vector" and "plasmid" are used interchangeably. However, the invention is intended to include other forms equivalently.

In the DNA fragments of the present invention, the segments of the expression cassette(s) are said to be "operationally associated" with one another. The DNA sequence encoding KPI peptides is positioned and oriented functionally with respect to the promoter, the DNA sequence encoding the S. cerevisiae αMF pre-pro sequence (including the DNA sequence encoding the αMF processing-site: lys-arg), and the transcription terminator. Thus, the polypeptide encoding segment is transcribed, under regulation of the promoter region, into a transcript

capable of providing, upon translation, the desired polypeptides. Because of the presence of the αMF pre-pro sequence, the expressed KPI product is found as a secreted entity in the culture medium. Appropriate reading frame positioning and orientation of the various segments of the expression cassette are within the knowledge of persons of ordinary skill in the art; further details are given in the Examples.

The DNA fragment provided by the present invention may include sequences allowing for its replication and selection in bacteria, especially E. coli. In this way, large quantities of the DNA fragment can be produced by replication in bacteria.

Methods of transforming methylotrophic yeast, such as, for example, Pichia pastoris, include the spheroplast technique, described by Cregg et al., 1985, in Mol. Cell. Biol. 5:3376 and U.S. Patent No. 4,879,231, or by the whole-cell lithium chloride yeast transformation system (Ito et al., 1984, Agric. Biol. Chem. 48:341), with modification necessary for adaptation to methylotrophic yeast, such as P. pastoris (See European Patent Application No. 312,934; U.S. Patent No. 4,929,535).

It is also possible to construct KPI expression vectors containing more than one copy of the KPI gene expression cassette (i.e., AOX1 promoter-αMF prepro-KPI gene AOX1 terminator). Such multicopy vectors enable the transfer of multiple KPI genes into the host genome on a single DNA fragment. The resulting transformants, containing, for example, two, four, six, or more, KPI gene expression cassettes integrated into their genomes, may then be capable of increased production of recombinant KPI.

In order to prepare a vector containing two copies of the KPI gene expression cassette, the

approximately 1700 bp BglII-BamHI fragment containing the expression cassette is isolated from a BglII-BamHI digest of pKPI200. The isolated fragment is then ligated to BamHI-digested pKPI200. Analysis of the restriction digests of the resulting plasmid containing two head-to-tail tandem repeats of the KPI gene expression cassette (rather than inverted repeats) enables verification of the correct two-copy plasmid.

In order to prepare a vector containing four copies of the KPI gene expression cassette, the two-copy plasmid is digested with BglII/BamHI, and the BglII-BamHI fragment containing two copies of the expression cassette is isolated. This BglII-BamHI fragment is then inserted back into the unique BamHI site of the two-copy plasmid. Thus, two additional KPI expression cassettes are added to the plasmid containing two copies of the cassette to yield a single plasmid containing four copies of the KPI gene expression cassette arranged in a head-to-tail fashion.

To construct a vector containing six copies of the KPI gene expression cassette, the two-copy vector is digested with BglII/BamHI, and the BglII-BamHI fragment containing two copies of the expression cassette is isolated. This BglII-BamHI fragment is then inserted into the unique BamHI site of the four-copy vector. Thus, two additional KPI gene expression cassettes are added to the plasmid containing four copies of the cassette to yield a single vector containing six copies of the KPI gene expression cassette arranged in a head-to-tail fashion.

Positive transformants are characterized by Southern blot analysis (Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring

Harbor Laboratory Press, Cold Spring Harbor, New York, USA) for the site of DNA integration; Northern blots (Maniatis, Op. Cit.;, R.S. Zitomer and B.D. Hall, 1976, J. Biol. Chem. 251:6320) for methanol-responsive KPI synthetic gene expression; and product analysis for the presence of secreted KPI peptides in the growth media.

Transformed strains, which are of the desired phenotype and genotype, are grown in fermentors. For the large-scale production of recombinant DNA-based products in methylotrophic yeast, a three-stage, high cell-density, fed batch fermentation system is normally the preferred fermentation protocol employed. In the first, or growth stage, expression hosts are cultured in defined minimal medium with an excess of a non-inducing carbon source (e.g., glycerol). When grown on such carbon sources, heterologous gene expression is completely repressed, which allows the generation of cell mass in the absence of heterologous protein expression. Next, a short period of non-inducing carbon source limitation growth is allowed to further increase cell mass and derepress the methanol responsive promoter. Subsequent to the period of growth under limiting conditions, methanol alone, or a limiting amount of a non-inducing carbon source plus methanol (in either case, referred to herein as "methanol excess fed-batch mode") are added in the fermentor, inducing the expression of the KPI synthetic gene driven by a methanol responsive promoter. This third stage is the so-called production stage.

The term "culture" means a propagation of cells in a medium conducive to their growth, and all subcultures thereof. The term "subculture" refers to a culture of cells grown from cells of another culture

(source culture), or any subculture of the source culture, regardless of the number of subculturings which have been performed between the subculture of interest and the source culture.

According to a preferred embodiment of the present invention, the heterologous protein expression system used for KPI production utilizes the promoter derived from the methanol-regulated AOX1 gene of P. pastoris, which is very efficiently expressed and tightly regulated. This gene can be the source of the transcription terminator as well. The presently preferred expression cassette comprises, operationally associated with one another, the P. pastoris AOX1 promoter, DNA encoding the S. cerevisiae αMF pre-pro sequence (including the DNA sequence encoding the αMF processing site: lys-arg), a DNA sequence encoding KPI, and a transcription terminator derived from the P. pastoris AOX1 gene. Preferably, two or more of such expression cassettes are contained on one DNA fragment, in head-to-tail orientation, to yield multiple expression cassettes on a single contiguous DNA fragment. The presently preferred host cells to be transformed with multiple expression cassettes are P. pastoris cells having at least one mutation that can be complemented with a marker gene present on a transforming DNA fragment. Preferably His4$^-$ (GS115) or Arg4$^-$ (GS190 NRRL Y-18014) auxotrophic mutant P. pastoris strains are employed.

The fragment containing one or more expression cassette(s) is inserted into a plasmid containing a marker gene complementing the host's defect, and optionally containing additional sequences such as bacterial marker genes, yeast sequences which direct vector integration, and the like. pBR322-based

plasmids, e.g., pAO815, are preferred. Insertion of one or more copies of the gene is also preferred.

The expressed KPI, which is preferably secreted into the culture medium, is purified to homogeneity, e.g., by chromatography. In a specific embodiment, the KPI is purified by cation exchange chromatography. However, other purification techniques known in the art can also be used, including affinity chromatography, e.g., on a support coupled with protease such as trypsin, size exclusion chromatography, and reverse-phase chromatography.

While in a specific embodiment high-level expression of KPI from APP is demonstrated, one of ordinary skill in the art would recognize that these same methods can be used for high level expression of a KPI with the same protease specificity as APP KPI. Preferably additional KPI species can be prepared by site-directed mutagenisis. Such KPI species incorporate the APP KPI amino acids in the active site of the KPI, in particular at P_2, P_1, P_1', and/or P_2'. Alternatively, an analog of the KPI of amyloid precursor protein with altered specificity can be prepared. KPIs for use in the therapeutic methods of the invention are described in detail in Section 5.2.1., infra.

5.2. THE USE OF KPI TO TREAT AMYLOIDOSIS AND PROTEASE-ASSOCIATED DISORDERS

The KPI of the present invention can be used in the treatment or prevention of diseases associated with amyloidosis, including but not limited to Alzheimer's disease, Down's syndrome, neuronal acid lipofuscinosis, cerebral amyloid angiopathy, hereditary cerebral hemorrhage with amyloidosis - Dutch type and Icelandic type and amyloidosis associated with head trauma, such as dementia

pugilistica. Although the present invention is not intended to be limited by any mechanism or mode of action, it is believed that the therapeutic effectiveness of KPI is in its ability to regulate proteolytic events in the processing of APP. The therapeutic efficacy of KPI peptide in the treatment or prevention of amyloidosis depends on administration of the peptide in an appropriate dose. As shown in an example (Section 9, infra), high doses of KPI (10 μM to 1 mM) appear to alter processing of the C-terminus of APP, thus an appropriate dose of KPI is below the threshold dose that appears to alter processing in this manner.

In another embodiment, appropriate doses of the KPI of the invention can be used in the treatment of coagulation disorders. The KPI inhibits the activity of proteases in the coagulation pathway even at relatively low doses, and has an anticoagulant effect. In particular, the KPI can be used to effectively inhibit Factor XIa activity in the presence or absence of heparin. Inappropriate coagulation has been associated with neurodegenerative disorders, such as HCHWA. HCHWA-(Dutch type) is characterized by recurrent strokes caused by extensive cerebral amyloid angiopathy. It is possible that in HCHWA (Dutch type) APP KPI, altered because of a point mutation (Levy et al., 1990, Science 248:1124-1126) functions inadequately in its inhibition of Factor XIa and thus invokes a tendency to develop hemorrhagic infarcts. Thus the present invention can provide therapeutic treatment for two pathological neurodegenerative disease mechanisms. Other coagulation disorders include but are not limited to stroke, embolisms, peripheral clotting, and cerebrovascular coagulation disorders.

Furthermore, proteolysis elicited by head trauma can be treated with appropriate doses of KPI.

In yet another embodiment, KPI of the invention can be used to treat pathological conditions involving proteolytic activity, such as acute pancreatitis, emphysema, cystic fibrosis, and the like.

5.2.1. KPIs FOR USE IN TREATMENT

According to the present invention, any KPI, e.g., such as those described in Section 2.2, supra, having protease inhibitory activity, such as but not limited to coagulation factors, trypsin, chymotrypsin or elastase inhibitory activity, can be used in the therapeutic methods of the invention. In particular, a KPI for use in the therapeutic methods of the invention when administered in an appropriate dosage range regulates proteases involved in the processing of APP. In one embodiment, the KPI includes the amino acids arginine, alanine, methionine and isoleucine at positions corresponding to P_1, P_1', P_2' and P_3' of the Kunitz-type reactive center sequences shown in Table I.

In a further embodiment, a peptide analog of KPI designed to enhance inhibition of certain types of proteases involved in processing of APP can be used in the therapeutic methods of the invention. As discussed in Section 5.4., infra, the present invention enables development of peptides capable of inhibiting proteases.

In a preferred embodiment, the APP KPI, having about 61 amino acids of the native sequence of KPI^+ APP (SEQ. ID NO. 2), is used. Preferably the KPI is obtained by high level expression in methylotrophic yeast, as described in Section 5.1., supra. The

abundant yield of KPI secreted from methylotrophic yeast provides a sufficient amount of KPI in an initially purer form free from fusion peptides for therapeutic use.

A KPI or KPI analogs produced in accordance with the invention may be tested for therapeutic potential. Suitable tests include but are not limited to one or more of the following: trypsin inhibitory activity; Factor XIa inhibitory activity -- with and without heparin; prolongation of clotting time; chymotrypsin inhibitory activity; the ability to complex with EGF binding protein; elastase inhibitory activity; and cathepsin G inhibitory activity. A particular KPI of KPI analog of the invention may be more or less effective in any of the foregoing tests, depending on its specificity. In a further embodiment, the KPI of the invention demonstrates the ability to regulate the proteolytic processing of APP. Regulation of APP processing can be assayed *in vivo* or *in vitro*, as described in Section 5.5., *infra*. In yet a further embodiment, the KPI of the inveniton can prolong clotting time in a clotting assay. In yet a further embodiment, the KPI can prevent or reduce the degree of damage to the brain after head trauma, e.g., in the model described in Section 5.6, *infra*.

5.2.2. ADMINISTRATION OF KPI TO PATIENTS

A therapeutically effective dose of KPI can be administered to a subject suffering from or suspected of suffering from a disease or condition associated with amyloidosis, proteolytic activity, a coagulation disorder or head trauma. As discussed in detail *infra*, therapeutic dosage ranges can be derived from *in vitro* or *in vivo* (animal model studies), or can be correlated with the level of soluble APP found

in serum or CSF. Preferably the subject is a mammal, and more preferably the subject is a human, although any animal can be treated according to the present invention.

In one embodiment, a therapeutically effective dose of the KPI of the invention is one that will reduce or terminate amyloidosis, and prevent further amyloid plaque damage. In a further embodiment, KPI is administered to maintain an in vivo concentration in the patient found to be effective to eliminate or reduce amyloidogenic A4 synthesis in vitro. While intraventricular, intracranial and intravenous injection are very effective forms of administration, and may be preferred for the treatment of amyloidosis, other modes can be employed, including but not limited to intramuscular, intraperitoneal, and subcutaneous injection, and oral, nasal and parenteral administration.

In another embodiment, the KPI of the invention can be administered to a subject for the treatment of a coagulation disorder. More particularly, the KPI of the invention can be used to treat inappropriate clotting disorders by inhibiting proteases involved in the coagulation pathway. Such proteases include but are not limited to Factor XIa. The KPI is administered to the subject, preferably a human subject, in an amount effective to inhibit the clotting disorder, which can be determined by in vitro clotting assays that measure prolongation of clotting time. Therapeutically effective dosages of KPI for the treatment of a coagulation disorder can be determined from in vitro or in vivo models. In a specific example (Section 8, infra), in vitro assays show that KPI inhibits Factor XIa. Preferably for the treatment of a coagulation disorder, the KPI is

administered intravenously or intra-arterially. Other modes can be employed, including but not limited to intramuscular, intraperitoneal, and subcutaneous injection, and oral, nasal and parenteral administration.

In yet another embodiment, the KPI of the invention can be administered to a subject suffering from head trauma. Although the present invention is not intended to be bound by any particular theory, it is believed that KPI inhibits damage caused by proteases that are release or activated after head truma or a head injury. A therapeutically effective amount of KPI for administration to a subject who has suffered head trauma is an amount effective to prevent brain damage associate with head trauma, which can be determined from in vivo models of head trauma, e.g., as described in section 5.6., infra. Alternatively, an effective amount of KPI may be an amount effective to inhibit proteases. Preferably the KPI is administered intracranially, intraventricularly, or intraveneously for the treatment of a head injury, but other modes of administration can also be used, such as but not limited to intramuscular, intraperitoneal, and subcutaneous injection, and oral, nasal and parenteral administration.

In yet another embodiment, a therapeutically effective amount of the KPI of the invention can be administered to a patient suffering from a pathological condition involving proteolysis, such as emphysema, cystic fibrosis, acute pancreatitis, and the like. Such a therapeutically effective amount can be determined from in vitro assay, e.g., of elastase inhibition.

The KPI of the invention can be combined with appropriate pharmaceutically acceptable carriers,

diluents and adjuvants. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium carbonate, magnesium stearate, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained-release formulations and the like. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain an effective therapeutic amount of the KPI together with a suitable amount of carrier so as to provide the form for proper administration to the patient.

The KPI of the present invention can be modified by attachment to soluble macromolecules such as proteins, polysaccharides, or synthetic polymers. For example, the peptide could be coupled to styrene-maleic acid copolymers (see, e.g., Matsumura and Maeda, 1986, Cancer Res. 46:6387), methacrylamide copolymers (Kopececk and Duncan, 1987, J. Controlled Release 6:315), or polyethylene glycol (PEG)(e.g., Hershfield and Buckley, 1987, N. Engl. J. Med. 316:589; Ho et al., 1986, Drug Metab. Dispos. 14:349;

Chua et al., 1988 Ann. Intern. Med. 109:114). The KPI may be specifically targeted by attachment to antibody, especially a monoclonal antibody. Such antibodies include but are not limited to chimeric, single chain, Fab fragments, and Fab expression libraries. In one embodiment the KPI is coupled to the macromolecule via a degradable linkage so that it will be released in vivo in its active form. In another embodiment, the KPI coupled to the macromolecule may be active.

In another embodiment, the KPI may be delivered in a vesicle, in particular a liposome (see Langer, 1990, Science 249:1527-1533; Treat et al., 1989, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365; Lopez-Berestein, ibid., pp. 317-327; see generally ibid.)

In yet another embodiment, the KPI can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980, Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321: 574). In another embodiment, polymeric materials may be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida, 1974; Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.) Wiley, New York 1984; Ranger and Peppas, 1983, J. Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351; Howard et al., 1989, J. Neurosurg. 71:105). In a preferred embodiment, a controlled release system may be placed next to the therapeutic target, thus requiring only a fraction of the systemic

dose (see, e.g., Goodson, 1984, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138). For example, the controlled release system can be implanted in the brain leptomeningeal vessels (leptomeninges). It will be recognized by one of ordinary skill in the art that a particular advantage of the invention is that KPI will not be subject to the problems of denaturation and aggregation associated with a larger, less structured protein, such as native APP held in the warm, moist environment of a body in a controlled release system.

Other controlled release systems are discussed in the review by Langer (1990, Science 249: 1527-1533).

Preferably, when administration of the KPI for the treatment, regulation or prevention of amyloidosis or proteolysis elicited by head trauma is via intravenous, subcutaneous, intraperitoneal, oral, nasal or parenteral administration, the KPI will include a delivery vehicle that potentiates transport across the blood-brain barrier, e.g., KPI can be coupled to a monoclonal antibody against the transferrin receptor (Friden et al., 1991, Proc. Nat'l. Acad. Sci. U.S.A. 88:4771-4775) which will bind preferentially to endothelial cells of the brain and enhance the delivery of KPI to the neuronal parenchyma. Preferably a system for delivering peptides into the CNS e.g., such as that described by Bodor, et al. (1992, Science 257:1698-1700) is used. However, one particular advantage of the KPI of the invention, and especially of a KPI peptide analog, is that these molecules will more readily cross the blood-brain barrier than the entire APP 751, or a larger protein containing KPI. The ability of KPI of the invention to cross the blood-brain barrier is

further enhanced by the degradation of the barrier, which becomes leaky during amyloidosis.

In another embodiment, the dosage of KPI is sufficient to maintain the levels of secreted APP/PN-2 in CSF or serum equivalent to those in CSF or serum of normal subjects as described for treatments using PN-2 or APP in International Patent Publication No. WO 91/16628, corresponding to U.S. Application Serial No. 07/513,786, filed April 24, 1990 by Van Nostrand et al., now abandoned, incorporated herein by reference in their entirety. WO 91/16628 also describes assays for monitoring the progress of amyloidosis, especially in Alzheimer's disease, and thus provides for monitoring therapeutic treatment.

5.3. PROBING AND ISOLATION OF SERINE-TYPE PROTEASES

The abundant amounts of KPI provided by the present invention can be used to probe for and to isolate serine-type proteases, especially proteases involved in processing of APP, coagulation, and brain damage following head trauma. In one embodiment, the KPI is detectably labeled, for example, and not by way of limitation, with a radioisotope, an enzyme, a fluorescent molecule, colloidal gold, or a latex bead. In another embodiment, presence of KPI, which can be indicative of the presence of a protease, is detected by binding a detectable secondary reagent, such as an antibody specific for KPI, provided that the second reagent does not compete with the protease for binding to the KPI. Detectable KPI can be used to detect proteases in situ, blotted on a membrane or support, e.g., by "Western" blotting, in solution using an "ELISA" or precipitation assays or by other methods

known in the art for detecting binding of ligand and receptor.

In another embodiment, a protease can be detected in vivo. In one embodiment, in vivo detection of protease, especially protease associated with processing of APP, coagulation, or damage due to head trauma with amyloidosis, provides a diagnostic indication of disease, since the level or amount of protease can be quantitatively or qualitatively determined. In one embodiment, the KPI is detectably labeled with a heavy-metal-chelate, a magnetic resonance imaging agent, or a radioisotope for in vivo detection.

In yet a further embodiment, the abundant KPI of the invention can be used to prepare affinity reagents for the isolation and characterization of proteases, especially proteases associated with processing of APP, coagulation, and damage following head trauma. According to the invention, KPI can be conjugated to a solid phase support, such as are commonly used in affinity purification. In particular, the KPI may be coupled to cyanogen bromide (CNBr)-activated Sepharose. The KPI-solid support conjugate can be used to isolate protease by affinity chromatography or affinity precipitation (i.e., analogous to immunoprecipitation).

Any serine protease that binds to, and preferably is inhibited by, KPI can be isolated according to the foregoing methods. The protease may be involved in a pathological disorder or condition. Preferably the protease is involved in the processing of APP to form, for instance, β-amyloid or soluble PN-2. In another aspect, the protease is involved in disorders of coagulation. In yet another embodiment, the protease is one that is released into the brain

after head trauma and is involved in the brain damage that follows head trauma. In a further embodiment, the protease is involve in a pathological condition such as cystic fibrosis, acute pancreatitis, emphysema and the like. These and other proteases may be distinguished based on relative affinity for binding KPI, or ability to hydrolyze APP or fragments of APP for example, or on the basis of physical-chemical properties such as molecular weight, sedimentation coefficient, iso-electric point, and solubility.

Once proteases involved in processing of APP, coagulation, or damage due to head trauma are isolated according to the present invention, the proteases can be studied. In particular, other protease inhibitors can be tested for their ability to selectively block the inappropriate proteolytic pathways, while not affecting the appropriate proteolytic pathways. For example, and although the present invention is not to be limited by any particular hypothesis or theory, KPI may differentially inhibit different proteases at differing doses. The dosage dependence of KPI inhibition of these different proteases may reflect the different affinity of KPI for each protease.

5.4. IDENTIFICATION OF KPI ANALOGS BASED ON KPI STRUCTURE

By providing an abundant source of KPI, the present invention enables quantitative structural determination of KPI. In particular, enough material is provided for NMR, IR, Raman, and ultraviolet, especially circular dichroism, spectral analysis. In particular, NMR provides very powerful structural analysis of native molecules in solution (Marion et al., 1983, Biochem. Biophys. Res. Comm. 1983 113:967-

974; Bax et al. 1985, J. Magn. Reson. 65:355-360; Kimura et al., 1980, Proc. Nat'l. Acad. Sci. U.S.A. 77:1681-1685).

In another embodiment, KPI crystals can be formed and analyzed by X-ray crystallography. More preferably, co-crystals of KPI and protease can be formed and analyzed. Even more preferably, KPI and the proteases associated with processing of APP, coagulation, or brain damage due to head trauma can be co-crystallized. Analysis of such co-crystals would provide detailed information about the structure of the protease inhibitor in its inhibitory state.

From the detailed structural analysis of KPI provided by the instant invention, peptide analogs of KPI of substantially lower molecular weight than KPI can be rationally designed. The structural analogs may be produced synthetically, or alternatively, when the design includes only natural amino acids, the analog may be produced recombinantly.

5.5. IN VITRO AND IN VIVO MODELS OF AMYLOIDOSIS INVOLVING KPI

The present invention provides an in vitro model for elucidation of APP processing and amyloidosis based on the production of PN-2 and β-amyloid protein by neuronal-type cells, preferably human neuronal-type cells. In a specific embodiment, neuroblastoma cells are used. Processing of APP can be followed in the presence of varying concentrations of, and absence of, KPI. The KPI can be added exogenously; preferably the KPI used is from a source that produces KPI in high levels, such as the yeast expression system of the present invention. Alternatively, an expression vector comprising a gene encoding KPI can be introduced into the neuronal-type cell for in situ expression. In one embodiment, an

expression vector comprising the KPI gene disclosed in SEQ ID NO. 3 can be used. In another embodiment, an expression vector comprising the native KPI gene (e.g., as described in Sinha et al., 1990, J. Biol. Chem. 265:8983-8985; Schilling et al., 1991, Gene 98:225-230), can be used. In a specific embodiment (Section 9, infra), the neuronal-type cell SHSY5Y, a human neuroblastoma cell line, that secretes high levels of APP, is used for an in vitro assay of amyloidosis. In another embodiment, IMR32 cells (ATCC CCl 127) can be used.

In another embodiment, a transgenic animal model, in which the transgenic animal is transgenic with an expression vector comprising a KPI gene, can be used as an in vivo model of APP processing and amyloidosis. Preferably, the KPI expression is under control of a tissue specific regulation system; more preferably, KPI expression is under control of a neuronal-type cell-specific regulation. That is, the KPI peptide will only be expressed by neuronal-type cells in the animal. The KPI expressed in a transgenic animal should also include a signal sequence for secretion from the cells, since it is believed that the relevant proteases are accessable extracellularly. Thus the effect of KPI expression in neuronal-type cells in vivo can be evaluated. One example of a transgenic animal model that can be adapted for expression of KPI is that reported by Quon et al. (1991, Nature 352:239-241). Other such models are well known in the art, and can be used for this invention.

In another embodiment, the ability of KPI to regulate APP processing can be tested in vivo. To this end APP 751 and APP 695 may be introduced into a mouse by intravenous or intraventricular injection, or

by expression in a transgenic animal model (e.g., Quon et al., 1991, supra). Both neuronal parenchymal and cerebrovascular amyloid deposition can be found in amyloidosis, thus the present assay relates to actual pathological conditions. Groups of mice that have been challenged with the APP 751 or APP 695, and control mice that have not been challenged, are treated with an appropriate dosage of the KPI of the invention or with a control lacking KPI. The treatment can be administered by intravenous, intracranial and/or intraventricular injection. The mice are sacrificed and the brain tissue is examined for amyloid deposits, i.e., amyloidosis. The amount of amyloid deposition will indicate the degree of involvement of KPI in the processing of APP, and in particular APP 751 and APP 695.

Moreover, the role of KPI in regulating processing of APP can be evaluated in vivo simply by injecting a wide range of doses of KPI into an animal, such as a mouse or rat. Injection can be intravenous, intracranial, and/or intraventricular. Both the KPI transgenic and KPI administration models provide for in vivo evaluation of KPI regualtion of proteases involved in APP processing.

5.6. HEAD INJURY MODEL

The present invention contemplates use of the high levels of KPI expressed by yeast in animal models of head injury. In particular, the KPI can be evaluated for its therapeutic benefit in the treatment of head injury. Any head injury model known in the art can be used to evaluate the therapeutic effectiveness of KPI. In specific embodiments, head injury in simians, dogs, cats, or other typical animal models can be used. As is well known in the art,

using routine experimentation, typical outcome measures of a head injury model include, but are not limited to, cardiovascular function; brain water and tissue cation content; regional cerebral blood flow; alterations in brain metabolism; neurological evaluation; evaluation of memory defects; and histopathological analysis. The data from one or more of these outcome measures can be obtained from normal control animals, injured animals, and injured animals that have been treated with the KPI of the invention, and the data compared to determine efficacy of the treatment and establish therapeutically effective doses of KPI for use in treatment. Preferably the KPI is administered intraventricularly, but any of the administration techniques discussed in Section 5.2.2., supra, can be used for the administration of the KPI.

In a preferred embodiment, the fluid percussion (FP) model of head injury will be employed using the anesthetized rat. This model was initially developed by Lindgren and Rinder (1969, Acta. Physiol. Scand. 76:340-351) in the rabbit and utilizes hydraulically-induced pressure changes to produce quantifiable, mechanical deformation of the brain.

The FP model yields a predictable degree of brain injury and simulates many aspects of head injury in humans. The technique produces pressure transients (20 msec) similar to those recorded in human cadaver skulls during sudden impact (Sullivan et al., 1976, J. Neurosurg. 45:520-534; Wei et al., 1980, Circ. Res. 46:37-47; Lindgren et al., 1966, Biophysik 3:164-180) as well as neurological signs of behavioral suppression resembling signs of unconsciousness in humans (Teasdale, et al., 1974, Lancet 81:84-85). Diminution or abolition of cerebrovascular responsiveness to changes in $PaCO_2$, similar to that

observed in the brain of injured humans, has also been reported in animals following FP injury (Wei et al., 1980, supra; Saunders, 1979, J. Neurosurg. 51:18-26; Lewelt, J. Neurosurg. 53:500-511). FP injury also results in a loss of cerebrovascular autoregulation (Lewelt, 1982, J. Neurosurg. 56:332-338; Fieschi et al., 1972, Eur. Neurol. 8:192-199) similar to that reported in brain injured patients (Sullivan et al., 1976, supra). In addition, centrally oriented FP brain injury results in brain stem pathologies (Rosenblum, 1981, Neurosurgery 9:613-620) reminiscent of those observed in animals after FP injury (Rosner et al., 1982, Proc. of the Fifth Int'l Symposium On Intracranial Pressure, Raven Press: New York, p. 154). More recently, it has been observed that both centrally and temporally oriented FP injury in the rat can be associated with edema, probably of vasogenic origin (McIntosh et al., 1990, Acta Neurochir. 51:263-264) and changes in regional blood flow (Yamakami and McIntosh, 1989, J. Cereb. Blood Flow Metab. 9:117-124; Yamakami and McIntosh, 1989, J. Cereb. Blood Flow Metab. 11:655-660).

Further details of this method may be found in several recent methodological papers (McIntosh et al., 1989, Neuroscience 28:233-244, Cortez et al., 1989 Brain Res. 462:271-282) where the neurological, neurochemical, and physiological responses to FP head injury in the rat are extensively characterized. FP produces initial generalized areflexia and subsequent comatose hypotonia without the presence of seizure activity, as well as anatomical, metabolic, and cerebrovascular changes similar to those reported in other species including rabbit, cat, and human. Moreover, data indicate that the model is sensitive to pharmacological intervention. The present method

produces a moderate-to-severe level of brain injury is produced, corresponding to 2.0-3.0 atm (atm = 14.7 lb/sq in).

At various intervals after FP brain injury, animals are randomly assigned to treatment with KPI or a control vehicle (equal volume). Pretreatment studies with KPI may also be permitted. The KPI is administered over a range of dosages in order to determine the most effective dose. After KPI treatment, animals are randomly assigned to the study groups.

The invention will be further understood by reference to the following examples, which are intended to be purely exemplary of the invention.

6. EXAMPLE: CONSTRUCTION OF KPI-EXPRESSION VECTORS

6.1. MATERIALS AND METHODS

6.1.1. CONSTRUCTION OF SYNTHETIC GENE

A synthetic DNA sequence encoding amino acid residues 285 through 345 of the amyloid precursor protein (Ponte et al., 1988, Nature 311:525-527) was constructed using eight overlapping oligonucleotides. SEQ ID NO. 1 provides the nucleotide and amino acid sequence of the final annealed synthetic gene. The synthetic gene sequence was formulated by back translation of amino acids 285-345 using a codon frequency computer program (University of Wisconsin Genetics Group (UWGCG)) in combination with consensus data generated from known gene sequences of the yeast Pichia pastoris. The synthetic gene sequence was designed to incorporate a HindIII site at its 5' end and both an EcoRI and BamHI site at its 3' end. The oligonucleotides were synthesized on an ABI 380A DNA

synthesizer by phosphoramidate chemistry, and were purified by HPLC on a DuPont 8800 reverse phase, Zorbax C8 column. The oligos were of the following sequences:

1. 5'-AGCTTGAGGTTGTTAGAGAGGTTTGTTCTGAGCAAGCTGAGACTG-3'
SEQ ID NO. 4

2. 5'-GTCCATGTAGAGCTATGATTTCTAGATGGTACTTCGACGTTACTGAGGGT-3'
SEQ ID NO. 5

3. 5'-AAGTGTGCTCCATTCTTCTACGGTGGTTGTGGTGGTAACAGAAACAACTT-3'
SEQ ID NO. 6

4. 5'-CGACACTGAGGAGTACTGTATGGCTGTTTGTGGTTCTGCTATTTAAGAATTCG-3'
SEQ ID NO. 7

5. 3'-GATCCGAATTCTTAAATAGCAGAACCACAAACAGCCATACA-5'
SEQ ID NO. 8

6. 3'-GTACTCCTCAGTGTCGAAGTTGTTTCTGTTACCACCACAACCACCGTAGA-5'
SEQ ID NO. 9

7. 3'-AGAATGGAGCACACTTACCCTCAGTAACGTCGAAGTACCATCTAGAAATC-5'
SEQ ID NO. 10

8. 3'-ATAGCTCTACATGGACCAGTCTCAGCTTGCTCAGAACAAACCTCTCTAACAACCTCA-5'
SEQ ID NO. 11

The eight oligonucleotides were annealed overnight without ligase. One hundred picomoles each of oligos 2, 3, 4, 6, 7, and 8 were kinased together in a 100 pl volume containing kinase buffer, 350 nM of ATP and 5 µl of polynucleotide kinase. The reaction was conducted at 37°C for 105 minutes. One µl of 0.5 M EDTA was added along with 100 pM each of oligos 1 and 5. The mixture was extracted with phenol/chloroform and the DNA precipitated with ethanol. The precipitated DNA was resuspended in 45 µl water, placed in a boiling water bath for 3 sec, and then held at 37°C for 5 min. Five µl of 10X HindIII buffer were added and the mixture was incubated overnight at 15°C. The annealed DNA was run on a 1.2% preparative agarose gel, and fragments of about 200 bp were isolated and resuspended in 40 µl water.

6.1.2. ATTACHMENT OF SECRETION SIGNALS

DNA encoding a yeast-specific secretion signal was attached to the synthetic gene utilizing plasmid pAO203. Plasmid pAO203 is a pUC18-based plasmid comprised of the S. cerevisiae alpha mating-factor (αMF) prepro signal sequence, including the lys-arg-(glu-ala)$_2$ processing sites, the GRF gene, and the P. pastoris AOX1 terminator.

Plasmid pAO203 was prepared as follows. The P. pastoris AOX1 transcription terminator was isolated from 20 μg of pPG2.0 (pPG2.0 is a BamHI-HindIII fragment of pG4.0 (NRRL accession no. 15868) + pBR322) by StuI digestion followed by the addition of 0.2 μg SalI linkers (GGTCGACC). The plasmid was subsequently digested with HindIII and the 350 bp fragment isolated from a 10% acrylamide gel and subcloned into pUC18 (Boehringer Mannheim) digested with HindIII and SalI. The ligation mix was transformed into JM103 cells (widely available) and Amp^R colonies were selected. The correct construction was verified by HindIII and SalI digestion, which yielded a 350 bp fragment, and was called pAO201.

Five μg of pAO201 was digested with HindIII, filled in using E. coli DNA Polymerase I Klenow fragment, and 0.1 μg of BglII linkers (GAGATCTC) were added. After digestion of the excess BglII linkers, the plasmid was reclosed and transformed into MC1061 cells. Amp^R cells were selected, DNA was prepared and the correct plasmid was verified by BglII, SalI double digests, yielding a 350 bp fragment, and by a HindIII digest to show loss of the HindIII site. This plasmid was called pAO202.

An alpha factor-GRF fusion was isolated as a 360 bp BamHI-PstI partial digest from pYSV201. Plasmid pYSV201 is the EcoRI-BamHI fragment of GRF-E-3

inserted into M13mp18 (New England Biolabs). Plasmid GRF-E-3 is described in European Patent Application No. 206,783. Twenty μg of pYSV201 plasmid were digested with BamHI and partially digested with PstI. To this partial digest were added the following oligonucleotides:

5' AATTCGATGAGATTTCCTTCAATTTTTACTGCA 3'
(SEQ ID NO. 12)

3' GCTACTCTAAAGGAAGTTAAAAATG 5'
(SEQ ID NO. 13)

Only the antisense strand of the oligonucleotide was kinase labelled so that the oligonucleotides did not polymerize at the 5'-end. After acrylamide gel electrophoresis (10%), the fragment of 385 bp was isolated by electroelution. This EcoRI-BamHI fragment of 385 bp was cloned into pAO202 which had been cut with EcoRI and BamHI. Routinely, 5 ng of vector cut with the appropriate enzymes and treated with calf intestine alkaline phosphatase, were ligated with 50 ng of the insert fragment. MC1061 cells were transformed, Amp^R cells were selected, and DNA was prepared. The resulting plasmid pAO203, was identified by cutting with EcoRI and BglII to yield a fragment of greater than 700 bp.

Approximately 1, 2 and 5 μl of a 1:10 dilution of the annealed and purified oligonucleotide described in Section 6.1.1., supra, and approximately 100 ng of pAO203, previously digested with BamHI and HindIII and purified on a 0.8% agarose gel, were ligated together in a standard ligation reaction. The ligation was transformed into E. coli MC1061 cells and Amp^R colonies were selected. Plasmid DNA from colonies containing correct plasmid demonstrated an approximately 450 bp band upon digestion with EcoRI and were called KPI100.

Twenty micrograms of CsCl-purified pKPI100 were digested with EcoRI in a 50 µl volume for 4 hr at 37°C and the digest stored at -20°C for two days. The approximately 450 bp EcoRI fragment was separated overnight on a 0.8% preparative gel. The fragment was resuspended in 40 µl of water and diluted 1:10. One, two, and five microliters of the 1:10 dilution were ligated into 1 µl of EcoRI-digested, dephosphorylated M13mp19 and the ligation mixture was used to transform competent E. coli JM103 cells. Twelve clear plaques were selected from the transformation plates, and grown overnight at 37°C in 3 ml LB medium. Plasmid minipreps of the evernight cultures were prepared by the alkaline lysis method, and the miniprep DNA was digested with PstI. A plasmid containing an insert of approximately 400-450 bp was designated KPI101 and was used to prepare a large template prep.

The PstI fragment was sequenced by the USB Sequenase Version 2.0 kit. The sequence was correct except for a one base deletion which was corrected as described in Section 6.1.3.

6.1.3. MUTAGENESIS

In vitro site-directed mutagenesis was performed to correct the single base pair deletion and to delete the DNA encoding the glu-ala-glu-ala spacer between the αMF-lys-arg sequence and the KPI coding sequence. The mutagenesis was performed as described by Zoller and Smith, 1983, Meth. Enzymol. 100:468. Plasmid KPI101 was used as template for the first mutagenesis, and plasmid KPI102, the product of the first mutagenesis, was used as template for the second mutagenesis.

The oligos used in the first round of mutagenesis, to delete the glu-ala-glu-ala encoding sequence, were as follows:

Mutagenizing oligo: GGGGTATCTTTGGATAAAAGAGAGGTTGTTAGAGAGGTTTGT
(SEQ ID NO. 14)

Screening oligo: GATAAAAGAGAGGTTGTT
(SEQ ID NO. 15)

Plasmid containing the correctly mutagenized insert was identified by hybridization to the screening oligonucleotide, and was called KPI102.

The second mutagenesis was performed to correct the one base pair deletion. The mutagenizing and screening oligos for this second mutagenesis were as follows:

Mutagenizing oligo: TACTTCGACGTTACTGAGGGTAAGTGTGCTCCATTCT
(SEQ ID NO. 16)

Screening oligo: GTTACTGAGGGTAAGTGT
(SEQ ID NO. 17)

Plasmids hybridizing with the screening oligo after the second round of mutagenesis were called KPI103. The complete EcoRI insert in KPI103 was sequenced with a Sequenase 2.0 kit and verified to be correct. The nucleotide sequence of the EcoRI fragment is shown in SEQ ID NO. 3.

6.1.4. CONSTRUCTION OF pKPI200 EXPRESSION VECTOR

Plasmid pKPI103 was digested with EcoRI and the 450 bp band was purified on a 1% agarose gel. Approximately 34.5 ng of fragment were ligated with 200 ng of pA0815 which had been digested with EcoRI and treated with calf intestinal alkaline phosphatase (see below for a description of pA0815 and Figure 1A). The ligation was transformed into MC1061 cells and Amp^R colonies were selected. Plasmid with the insert in the proper orientation yielded a 930 bp band upon

digestion with XbaI and was called pKPI200. Plasmid pKPI200 is shown in Figure 1B.

Plasmid pAO815 was constructed by mutagenizing plasmid pAO807 (described below) to change the ClaI site downstream of the AOX1 transcription terminator in pAO807 to a BamHI site. The oligonucleotide used for mutagenizing pAO807 had the following sequence: 5' GAC GTT CGT TTG TGC GGA TCC AAT GCG GTA GTT TAT 3'. The mutagenized plasmid was called pAO807-Bam. Plasmid pAO804 (also described below) was digested with BglII and 25 ng of the 2400 bp fragment were ligated to 250 ng of the 5400 bp BglII fragment from BglII-digested pAO807-Bam. The ligation mix was transformed into MC1061 cells and the correct construct was verified by digestion with Pst/BamHI to identify 6100 and 2100 bp sized bands. The correct construct was called pAO815. The restriction map of the expression vector pAO815 is shown in Figure 1A.

Plasmid pAO807 was prepared by the following procedure.

A. Preparation of f1-ori DNA: f1 bacteriophage DNA (50 µg) was digested with 50 units of RsaI and DraI (according to manufacturer's directions) to release the ~458 bp DNA fragment containing the f1 origin of replication (ori). The digestion mixture was extracted with an equal volume of phenol:chloroform (v/v) followed by extracting the aqueous layer with an equal volume of chloroform. The DNA in the aqueous phase was precipitated by adjusting the NaCl concentration to 0.2M and adding 2.5 volumes of absolute ethanol. The mixture was allowed to stand on ice (4°C) for 10 minutes and the DNA precipitate was collected by centrifugation for 30 minutes at 10,000 x g in a microfuge at 4°C.

The DNA pellet was washed 2 ~~times~~ with 70% aqueous ethanol. The washed pellet was vacuum dried and dissolved in 25 µl of TE buffer. This DNA was electrophoresed on 1.5% agarose gel and the gel portion containing the ~458 bp f1-ori fragment was excised out and the DNA in the gel was electroeluted onto DE81 (Whatman) paper and eluted from the paper in 1M NaCl. The DNA solution was precipitated as detailed above and the DNA precipitate was dissolved in 25 µl of TE buffer (f1-ori fragment).

B. Cloning of f1-ori into DraI sites of pBR322: pBR322 (2 µg) was partially digested with 2 units DraI (according to manufacturer's instructions). The reaction was terminated by phenol:chloroform extraction followed by precipitation of DNA as detailed in step A above. The DNA pellet was dissolved in 20 µl of TE buffer. About 100 ng of this DNA were ligated with 100 ng of f1-ori fragment (step A) in 20 µl of ligation buffer by incubating at 14°C for overnight with 1 unit of T4 DNA ligase. The ligation was terminated by heating to 70°C for 10 minutes and then used to transform E. coli strain JM103. Amp^R transformants were pooled and superinfected with helper phage R408. Single stranded phage was isolated from the media and used to reinfect JM103. Amp^R transformants contained pBRf1-ori, which contains f1-ori cloned into the DraI sites (nucleotide positions 3232 and 3251) of pBR322.

C. Construction of plasmid pAO807: pBRf1-ori (10 µg) was digested for 4 hours at 37°C with 10 units each of PstI and NdeI. The digested DNA was phenol:chloroform extracted, precipitated and dissolved in 25 µl of TE buffer as detailed in step A above. This material was electrophoresed on a 1.2% agarose gel and the NdeI-PstI fragment (approximately

0.8 kb) containing the f1-ori was isolated and dissolved in 20 µl of TE buffer as detailed in step A above. About 100 ng of this DNA were mixed with 100 ng of pAO804 (described hereinafter) that had been digested with PstI and NdeI and phosphatase-treated. This mixture was ligated in 20 µl of ligation buffer by incubating overnight at 14°C with 1 unit of T4 DNA ligase. The ligation reaction was terminated by heating at 70°C for 10 minutes. This DNA was used to transform E. coli strain JM103 to obtain pAO807.

Plasmid pAO804 employed in the above procedure was constructed as follows: Plasmid pBR322 was modified to eliminate the EcoRI site and insert a BglII site into the PvuII site. pBR322 was digested with EcoRI, the protruding ends were filled in with Klenow fragment of E. coli DNA polymerase I, and the resulting DNA was recircularized using T4 ligase. The recircularized DNA was used to transform E. coli MC1061 to ampicillin-resistance and transformants were screened for having a plasmid of about 4.37 kpb in size without an EcoRI site. One such transformant was selected and cultured to yield a plasmid, designed pBR322ΔRI, which is pBR322 with the EcoRI site replaced with the sequence (SEQ ID NO. 19):

5'-GAATTAATTC-3'

3'-CTTAATTAAG-5'

pBR322ΔRI was digested with PvuII and the linker, of sequence:

5'-CAGATCTG-3'

3'-GTCTAGAC-5'

was ligated to the resulting blunt ends employing T4 ligase. The resulting DNAs were recircularized, also with T4 ligase, and then digested with BglII and again recircularized using T4 ligase to eliminate multiple BglII sites due to ligation of more than one linker to

the PvuII-cleaved pBR322ΔRI. The DNAs, treated to eliminate multiple BglII sites, were used to transform E. coli MC1061 to ampicillin-resistance. Transformants were screened for a plasmid of about 4.38 kbp with a BglII site. One such transformant was selected and cultured to yield a plasmid, designated pBR322ΔRIBGL, for further work. Plasmid pBR322ΔRIBGL is the same as pBR322RI except that pBR322ΔRIBGL has the sequence (SEQ ID NO. 20):

5'-CAGCAGATCTGCTG-3'

3'-GTCGTCTAGACGAC-5'

in place of the PvuIII site in pBR322ΔRI.

pBR322ΔRIBGL was digested with SalI and BglII and the large fragment (approximately 2.97 kbp) was isolated. Plasmid pBSAGI5I, which is described in European Patent Application Publication No. 0 226 752, was digested completely with BglII and XhoI and an approximately 850 bp fragment from a region of the P. pastoris AOX1 locus downstream from the AOX1 gene transcription terminator (relative to the direction of transcription from the AOX1 promoter) was isolated. The BglII-XhoI fragment from pBSAGI5I and the approximately 2.97 kbp SalI-BglII fragment from pBR322ΔRIBGL were combined and subjected to ligation with T4 ligase. The ligation mixture was used to transform E. coli MC1061 to ampicillin-resistance and transformants were screened for a plasmid of the expected size (approximately 3.8 kbp) with a BglII site. This plasmid was designated pAO801. The overhanging end of the Sal1 site from the pBR322ΔRIBGL fragment was ligated to the overhanging end of the XhoI site on the 850 bp pBSAGI5I fragment and, in the process, both the SalI site and the xhoI site in pAO801 were eliminated.

Plasmid pBSAGI5I was then digested with ClaI and the approximately 2.0 kbp fragment was isolated. The 2.0 kbp fragment has an approximately 1.0-kbp segment which comprises the P. pastoris AOX1 promoter and transcription initiation site, an approximately 700 bp segment encoding the hepatitis B virus surface antigen ("HBsAg") and an approximately 300 bp segment which comprises the P. pastoris AOX1 gene polyadenylation signal and site-encoding segments and transcription terminator. The HBsAg coding segment of the 2.0 kbp fragment is terminated, at the end adjacent to the 1.0 kbp segment with the AOX1 promoter, with an EcoRI site and, at the end adjacent the 300 bp segment with the AOX1 transcription terminator, with a StuI site, and has its subsegment which codes for HBsAg oriented and positioned, with respect to the 1.0 kbp promoter-containing and 300 bp transcription terminator-containing segments, operatively for expression of the HBsAg upon transcription from the AOX1 promoter. The EcoRI site joining the promoter segment to the HBsAg coding segment occurs just upstream (with respect to the direction of transcription from the AOX1 promoter) from the translation initiation signal-encoding triplet of the AOX1 promoter.

For more details on the promoter and terminator segments of the 2.0 kbp, ClaI-site-terminated fragment of pBSAGI5I, see European Patent Application Publication No. 226,846 and Ellis et al., 1985, Mol. Cell Biol. 5:1111.

Plasmid pAO801 was cut with ClaI and combined for ligation using T4 ligase with the approximately 2.0 kbp ClaI-site-terminated fragment from pBSAGI5I. The ligation mixture was used to transform E. coli MC1061 to ampicillin resistance, and

transformants were screened for a plasmid of the expected size (approximately 5.8 kbp), which, on digestion with ClaI and BglII, yielded fragments of about 2.32 kbp (with the origin of replication and ampicillin-resistance gene from pBR322) and about 1.9 kbp, 1.48 kbp, and 100 bp. On digestion with BglII and EcoRI, the plasmid yielded an approximately 2.48 kbp fragment with the 300 bp terminator segment from the AOX1 gene and the HbsAg coding segment, a fragment of about 900 bp containing the segment from upstream of the AOX1 protein encoding segment of the AOX1 gene in the AOX1 locus, and a fragment of about 2.42 kbp containing the origin of replication and ampicillin resistance gene from pBR322 and an approximately 100 bp ClaI-BglII segment of the AOX1 locus (further upstream from the AOX1-encoding segment than the first mentioned 900 bp EcoRI-BglII segment). Such a plasmid had the ClaI fragment from pBSAGI5I in the desired orientation. If the fragment was in the opposite undesired orientation, digestion of the plasmid with EcoRI/BglII would yield EcoRI-BglII fragments of about 3.3 kbp, 2.38 kbp and 900 bp.

One of the transformants harboring the desired plasmid, designated pAO802, was selected for further work and was cultured to yield that plasmid. The desired orientation of the ClaI fragment from pBSAGI5I in pAO802 had the AOX1 gene in the AOX1 locus oriented correctly to lead to the correct integration into the P. pastoris genome at the AOX1 locus of linearized plasmid made by cutting at the BglII site at the terminus of the 800 bp fragment from downstream of the AOX1 gene in the AOX1 locus.

pAO802 was then treated to remove the HBsAg coding segment terminated with an EcoRI site and a

StuI site. The plasmid was digested with StuI and a linker of sequence:

5'-GGAATTCC-3'

3'-CCTTAAGG-5'

was ligated to the blunt ends using T4 ligase. The mixture was then treated with EcoRI and again subjected to ligating using T4 ligase. The ligation mixture was used to transform E. coli MC1061 to ampicillin-resistance and transformants were screened for a plasmid of the expected size (5.1 kbp) with EcoRI-BglII fragments of about 1.78 kbp, 900 bp, and 2.42 kbp and BglII-ClaI fragment of about 100 bp, 2.32 kbp, 1.48 kbp, and 1.2 kbp. This plasmid was designated pAO803. A transformant with the desired plasmid was selected for further work and was cultured to yield pAO803.

Plasmid pAO804 was then made from pAO803 by inserting, into the BamHI site from pBR322 in pAO803, an approximately 2.75 kbp BglII fragment from the P. pastoris HIS4 gene. See, e.g., Cregg et al, Mol. Cell. Biol. 5:3376 (1985) and European Patent Application Publication Nos. 180,899 and 188,677. pAO803 was digested with BamHI and combined with the HIS4 gene-containing BglII site-terminated fragment and the mixture subjected to ligation using T4 ligase. The ligation mixture was used to transform E. coli MC1061 to ampicillin-resistance and transformants were screened for a plasmid of the expected size (7.85 kbp), which is cut by SalI. One such transformant was selected for further work, and the plasmid it harbors was designated pAO804.

pAO804 has one SalI-ClaI fragment of about 1.5 kbp and another of about 5.0 kbp and a ClaI-ClaI fragment of 1.3 kbp; this indicates that the direction of transcription of the HIS4 gene in the plasmid is

the same as the direction of transcription of the ampicillin resistance gene and opposite the direction of transcription from the AOX1 promoter.

The orientation of the HIS4 gene in pAO804 is not critical to the function of the plasmid or of its derivatives with cDNA coding segments inserted at the EcoRI site between the AOXI promoter and terminator segments. Thus, a plasmid with the HIS4 gene in the orientation opposite that of the HIS4 gene in pAO804 would also be effective for use in accordance with the present invention.

6.1.5. CONSTRUCTION OF pKPIAV.21 EXPRESSION VECTOR

Plasmid pKPIAV.21 contains a synthetic gene encoding an analog of the KPI domain of APP in which the codon for the P_1 Arg has been replaced with a codon for Ile. Plasmid pKPIAV.21 was constructed as follows.

The synthetic KPI gene contained in pKPI200 was mutagenized using a polymerase chain reaction (PCR)-based site-directed mutagenesis procedure (Bowman et al., 1990, Technique 2:254-260) to change the codon for amino acid 17. The procedure consisted of two rounds of PCR amplification. The first round used the mutagenizing oligonucleotide

CTGAGACTGGTCCCATGTGTCGCGATGATTTCTAGATGGTACT

as the 5' primer (SEQUENCE ID NO. 23), and the oligonucleotide

GATTAGAATCTAGCAAGACC

as the 3' primer (SEQUENCE ID NO. 24) and pKPI200 as the template. The second round used oligonucleotide

CATAATTGCGACTGGTTCC

for the 5' primer (SEQUENCE ID NO. 25), and the same oligonucleotide for the 3' primer (SEQUENCE ID NO. 24) and an aliquot from the first amplification containing

the read-through product as the template. The oligonucleotide used as the 3' primer corresponds to nucleotides 77-58 (containing an AgeI site) in the 3' AOX1 termination region of pA0815 (see Figure 1A) and the 5' oligonucleotide used in the second round of PCR corresponds to nucleotides 7613-7631 (containing a HindIII site) in the 5' AOX1 promoter region of pA0815 (see Figure 1A). The mutagenizing oligonucleotide corresponds to the nucleotides encoding essentially amino acids 11-25 of KPI with four nucleotide differences: the nucleotides for the codon for amino acid 17, AGA (Arg codon), were replaced with GTC (Val codon) and the thymidine nucleotide located three nucleotides 3' of the Val codon was replaced with a guanine. The replacement of the codon for amino acid 17 was intended to modify the KPI-coding sequence such that it encoded a Val instead of an Arg for amino acid 17. The T-G change was included to insert a diagnostic NruI site without altering the amino acid coded for by the codon containing the nucleotide.

The products of the second round of PCR amplification were digested with HindIII and AgeI and ligated into HindIII/AgeI-digested pA0815. The resulting plasmids were analyzed for the diagnostic NruI site and were sequenced to confirm the mutation. The *in vitro* mutagenesis produced a 60-100% yield of mutagenized plasmid containing the diagnostic restriction site. Sequence analysis revealed a high rate of error in various regions of the PCR products as might occur due to poor fidelity of the Taq polymerase. One of the plasmids, pKPIAV.21, generated through the mutagenesis contained a correct sequence except that the mutation at codon 17 encoded an isoleucine instead of the intended valine.

6.1.6. TRANSFORMATION AND SELECTION OF KPI EXPRESSION STRAINS OF P. PASTORIS

Plasmids pKPI200 and pKPIAV.21 were digested with StuI and 10 μg of linearized plasmid were transformed into GS115 cells by the LiCl method of transformation (Ito et al., 1984, Agri. Biol. Chem. 48:341), with modification necessary for adaptation to methylotrophic yeasts, such as P. pastoris (see U.S. Patent No. 4,929,555 by Cregg et al., May, 1990). GS115 is a His⁻ strain of Pichia pastoris, deposited with the ATCC, assigned accession number 20864, and deposited with the NRRL, assigned accession number Y-15851. Linearization of pKPI200 at the StuI site directs the DNA to preferentially integrate at the HIS4 locus.

6.1.7. CHARACTERIZATION OF TRANSFORMANTS

Transformants were initially screened for histidine prototrophy. The integration event in cells transformed with pKPI200 was characterized in several of the histidine prototrophs by analysis of three separate Southern hybridizations. For two of the Southern hybridizations genomic DNA of the transformants was digested with BglII, electrophoresed through a 0.75% agarose gel, and then transferred to nitrocellulose filters. One filter was probed with plasmid pAO803 (See Section 6.1.2, supra) to determine if the transformants contained an intact AOX1 locus in addition to plasmid DNA. The other filter was probed with pYM4 (described in Cregg et al., 1985, Mol. Cell. Biol. 5:3376-3385 and obtained by digesting PYJ30 (NRRL accession number B-15890) with ClaI and religating the ends), a plasmid containing the P. pastoris HIS4 gene, to determine if the plasmid integrated into the HIS4 locus. The third Southern

evaluated StuI-digested DNA probed with the EcoRI fragment of pKPI200, to determine if the KPI expression cassette integrated in an intact form.

The Southern analyses indicated that transformants G+KPI200S1, G+KPI200S3, and G+KPI200S4 all contained one copy of intact plasmid pKPI200 at the HIS4 locus.

Twenty-four additional histidine prototrophs that had been transformed with pKPI200 were screened for high-level KPI production during growth in shake flasks. The 24 colonies were streaked out onto plates containing YNB-glucose (i.e., 0.67% yeast nitrogen base without amino acids; 2% glucose; 2% agar), grown for two days at 30°C, and then stored at 4°C. The plates were used to inoculate flasks containing approximately 40 ml of phosphate-buffered YNB medium (11.5 g/l KH_2PO_4, 2.66 g/l K_2HPO_4, 0.67% yeast nitrogen base, pH 5) containing 0.2% glycerol and 1% methanol. After 2 days growth at 30°C, broth was removed from the flasks and assayed for trypsin inhibitory activity (see Section 6.1.8). Broth from the shake flask containing strain G+KPI200S20 showed the highest level of trypsin inhibitory activity. Based on this result, it is likely that strain G+KPI200S20 contains at least two copies of vector pKPI200 which provide for correspondingly higher levels of expression of KPI than strains that integrated a single copy of PKPI200, e.g., strains G+KPI200S1, G+KPI200S3 and G+KPI200S4. Such multicopy integration events during transformation of P. pastoris have been found to occur at greater than 10% frequency. Therefore, in cases in which production of heterologous protein in P. pastoris is gene copy number dependent (i.e., the level of heterologous protein expression increases with increasing numbers of the heterologous gene

contained in the expression strain), it usually is possible to identify at least one multi-copy transformant by screening 20 transformants in this manner for high-level production of heterologous protein.

6.1.8. GROWTH OF KPI EXPRESSION STRAINS IN ONE AND TEN LITER FERMENTATIONS

Pichia pastoris transformed with plasmid pKPI200 were used for high-level expression of the recombinant polypeptide in one and ten liter fermentations. Pichia pastoris strain SMD1189, transformed with pKPIAV.21, was grown in one-liter fermentations. Pichia pastoris strains were grown in 2- and 15-liter fermentor vessels in order to accommodate the increase in volume during the induction phase of the fermentation. Because Pichia pastoris is grown to such a high cell density, the cell mass accounts for as much as 40% of the total volume in the fermentor at the end of a typical fermentation. Therefore, in a one-liter fermentation conducted in a 2-liter vessel, there will frequently be a total final culture volume of approximately 1.5 liters, of which approximately 750-1000 ml will be cell-free broth. Similarly, in a 10-liter fermentation conducted in a 15-liter vessel, approximately 5-6 liters of the total final volume is cell-free broth.

6.1.8.1. ONE-LITER SCALE

Run 1040: G+KPI200S3
Run 1042: G+KPI200S4
Run 1043: G+KPI200S1
Run 1062: G+KPI200S20
Run 1153: SMD1189

Media employed in fermentations described herein had the compositions shown in Table II.

TABLE IIA

COMPOSITIONS OF 10X BASAL SALTS

Chemical	Grams/liter
Phosphoric acid, 85%	42.0 ml
Calcium Sulfate. $2H_2O$	1.8
Potassium Sulfate	28.6
Magnesium Sulfate.$7H_2O$	23.4
Potassium Hydroxide	6.5

TABLE IIB

COMPOSITIONS OF PTM_1 TRACE SALTS

Chemical	Grams/liter
Cupric Sulfate.5H20	6.0
Sodium Iodide	0.08
Manganese Sulfate.H_2O	3.0
Sodium Molybdate.$2H_2O$	0.2
Boric Acid	0.02
Cobalt Chloride	0.5
Zinc Chloride	20.0
Ferrous Sulfate.$7H_2O$	65.0
Biotin	0.2
Sulfuric Acid	5.0 ml

Expression of the KPI polypeptide in strains G+KPI200S1, G+KPI200S3, G+KPI200S4 and G+KPI200S20 was analyzed in one-liter fermentation runs 1043 and 1040, 1042 and 1062, respectively. The one-liter fermentations were conducted as follows:

The fermentors were autoclaved with one liter of medium containing 500 ml of 10X basal salts, 2% glycerol, and the remainder deionized water. After cooling, 3 ml of PTM_1 trace salts were added and the pH was adjusted to between 4 and 5 by adding NH_4OH. During the fermentations, NH_4OH was used to maintain the pH and as a nitrogen source. Strucktol J673

antifoam (Strucktol Co., Stow, OH) was used to control foaming; temperature was maintained at 30°C, and agitation was adjusted to maintain the dissolved oxygen concentration above 20% of air saturation.

The batched fermentors were inoculated with 40 to 55 ml inoculum and the cell mass was allowed to increase until glycerol was exhausted. After glycerol exhaustion a feed of glycerol was started at 12 ml/h for four hours to derepress the enzymes in the methanol pathway and further increase cell density. When the glycerol feed ended, a methanol feed was initiated at 2 ml/h to induce production of KPI. After 3 h at 2 ml/h initial feed rate, the methanol feed rate was increased to 6 ml/h. After approximately 15 h at 6 ml/h, the methanol feed rate was increased to 10 ml/h.

Runs 1040 and 1042 were harvested 47 h after induction with methanol feed, run 1043 was harvested 28 h after induction, and run 1062 was harvested 49 h after induction. The final cell concentrations in runs 1040, 1042, 1043 and 1062 were 312 mg/ml, 314 mg/ml, 218 mg/ml, and 288 mg/ml, respectively.

6.1.8.2. TEN-LITER SCALE

Run 1044: G+KPI200S3
Run 1045: G+KPI200S3
Run 1052: G+KPI200S3
Run 1083: G+KPI200S20

Runs 1044, 1045 and 1052 were conducted with transformed P. pastoris G+KPI200S3 in ten liter fermentations. The ten-liter fermentations were conducted in a manner similar to the one-liter fermentations, with differences in volumes of medium components and rates of feed as follows. The

fermentors were charged with 4L 10X basal salts and 400 g glycerol, and sterilized. After sterilization, 26 ml of PTM_1 trace salts were added. The fermentors were inoculated with 500 ml inoculum. After glycerol exhaustion, a glycerol feed was started for 4 h at 120 ml/h. After the glycerol feed was terminated, the methanol feed was started at about 20 ml/h and increased in two steps, first after 3-4 h to 60 ml/h and then after 2-12 h to 100 ml/h.

Run 1044 was harvested 43 h after induction with methanol feed, run 1045 was harvested 40 h after induction and run 1052 who harvested 42 h after induction. The final cell concentrations were 501 mg/ml, 584 mg/ml, and 524 mg/ml runs 1044, 1045 and 1052, respectively.

Run 1083 was conducted with strain G+KPI200S20 in a 15-liter vessel. The fermentation was initiated and conducted in the same fashion as runs 1044, 1045 and 1052, except that at 41 h after methanol induction, 5 1 of broth were removed from the fermentor to make room for continued methanol feeding. After removal of the broth, the fermentation was allowed to continue for an additional 26 h. Shortly prior to harvest, the pH of the broth was decreased to 2.9. The final cell concentration was 544 mg/ml.

6.1.9. PRODUCT ANALYSIS

A trypsin inhibitory activity assay was performed to assay the protease inhibitory activity of the recombinant KPI domain. Samples (25 - 50 ml) of whole broth were removed from the fermentation runs and centrifuged at 4000 rpm for 10 min at 4°C to remove cells. The cell-free broth then was assayed for its ability to inhibit trypsin-induced cleavage of the chromogenic substrate benzoyl-L-arginine

p-nitroanilide (Sigma, St. Louis, MO). The assay was conducted as follows:

One hundred or 200 µl of a 1 mg/ml trypsin solution in incubation buffer (50 mM Tris-HCl, pH 8.0, 10 mm $MgCl_2$, 10 mm $CaCl_2$) were incubated in a final volume of 1 ml with 10 µl to 200 µl of test sample. The remainder of the volume was made up with 600 to 890 µl of incubation buffer.

The tubes were incubated at room temperature for 5 to 30 min (incubation time had no effect on the assay results), and then assayed for trypsin hydrolytic activity. To a quartz cuvette having a 1 cm path length were added 900 µl of incubation buffer and 50 µl of benzoyl L-arginine p-nitroanilide substrate (200 mM substrate in DMSO). Next, 50 µl of each of the KPI-trypsin solutions were separately added to a cuvette and mixed by inversion. The absorbance was measured over two or three minutes ("time run" function in kinetics mode) in a UV-vis spectrophotometer which had been blanked against incubation buffer at 410 nm.

The percentage inhibition was calculated as the decrease in slope of a linear correlation fit of A_{280} vs. time. In some cases the amount of inhibition was compared to that caused by a known amount of standard aprotinin analog.

A human neutrophil elastase inhibitory assay was performed to evaluate the inhibitory properties of the purified KPI analog (see Section 8, infra, for the purification procedure) produced by Pichia strain SMD1189 transformed with pKPIAV.21. Assays with the chromogenic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide were conducted in a buffer of 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 containing 0.1% BSA. Human neutrophil elastase (7 nM) was incubated for 20

min at 25°C with increasing amounts of KPI analog (1.75 nM, 3.5 nM, 7 nM, 14 nM). The residual elastase activity was measured at 405 nm in the presence of 560 μM chromogenic substrate.

6.2. RESULTS

Cell-free broth from three one-liter and two ten-liter fermentations (see Sections 6.1.7.1 and 6.1.7.2, supra) was evaluated for its trypsin inhibitory activity as described above. The results of these evaluations are shown in Table III.

TABLE III.

TRYPSIN INHIBITORY ACTIVITY OF RECOMBINANT KPI

Sample	Sample Volume	Trypsin volume	Percent inhibition
negative control[1]	100 μl	100 μl	0
Run 1040	100 μl	100 μl	46
Run 1042	100 μl	100 μl	54
Run 1043	200 μl	100 μl	68
Run 1044	20 μl	100 μl	44
Run 1045	20 μl	100 μl	49

[1] The negative control was cell-free broth from GS115 cells transformed with pIGF816, an expression vector that directs secretion of human insulin-like growth factor.

These data demonstrated that strains G+KPI200S1, G+KPI200S3, and G+KPI200S4 secreted active KPI into the fermentation broth. The lower trypsin inhibition shown by the run 1043 sample despite the

larger sample volume indicates that this fermentation of strain G+KPI200S produced less KPI, probably due to the starter induction time of the fermentation. The lower volumes of samples of run 1044 and 1045 broth that were able to inhibit trypsin activity by approximately 50% indicate that these 10 l fermentations produced higher concentrations of KPI than the 1-liter fermentations.

The results of elastase inhigitory assays of KPI analog purified from broth of run 1153 revealed that the analog is a potent inhibitor of this enzyme (K_i = 1.2 +/- 0.1 x 10^{-9} M).

Table IV below compares the results of 1- and 10-liter fermentations of strains G+KPI200S3 and G+KPI200S20 and MSD1189.

TABLE IV

KPI PRODUCTION

Fermentation Run Designation	Strain	Fermentor Size	KPI Concentration in Cell Free Broth
		(l)	(mg/l)
1040	G+KPI200S3	2	200[1]
1052	G+KPI200S3	15	360[1]
1062	G+KPI200S20	2	500[1]
1083	G+KPI200S20	15	1200[1]

[1] Quantitation by trypsin inhibitory activity.
[2] Quantitation by HPLC.

The KPI concentration in the cell-free broth of runs 1040, 1052, 1062 and 1083 was determined by the trypsin inhibitory assay (see Section 6.1.9.) and comparison of the inhibitory activity of the samples to the inhibitory activity of known amounts of purified Pichia-produced KPI (see section 8.1.1) that

were quantitated based on amino acid composition data (see Section 8.2.1). The data in Table IV show that KPI production at the 10-liter scale was higher than that at the 1-liter scale, and production by strain G+KPI200S20 was greater than that by strain G+KPI200S3. The higher productivity at the 10-liter scale compared to the 1-liter scale is a function of the fermentation protocol. At the 10-liter scale, proportionately more methanol and less water is added to the fermentor so that both cell concentration and product concentration are elevated relative to these concentrations in 1-liter fermentations. This is particularly true of run 1083 in which 5 1 of broth was removed after 41 h of induction to allow addition of more methanol, whereas run 1052 was harvested after 42 h of induction. However, after 41 h of induction in run 1083 with strain G+KPI200S20, the KPI concentration was already 800 mg/l, or more than twice the harvest KPI concentration of run 1052 with strain G+KPI200S3. Thus, it is clear that G+KPI200S20 is a superior expression strain, apparently due to the presumed presence of multiple copies of the expression vector integrated into the strain.

The KPI concentration in the cell-free broth of run 1153 was determined by HPLC using routine procedures and using purified Pichia-produced KPI as a standard.

7. EXAMPLE: PROTEIN SEQUENCE OF KPI EXPRESSED IN YEAST

Samples (5 ml) of cell-free broth from one-liter runs 1040 and 1042 were diluted in three volumes of 20 mM acetic acid and applied to a 0.3 ml cation exchange column (Spherodex M; IBF, Columbia, MD). The column was washed with 1 ml of 20 mM acetic

acid at room temperature. The bound protein was eluted with 2 ml of 1M NaCl in 50 mM NaOAc, pH 6. The trypsin inhibitory activity of eluates from columns that had been loaded with cell-free broth from runs 1040 and 1042 was assayed. The trypsin inhibitory activity was recovered in the saline eluate, as evidenced by the ability of 50 µl of the eluate to inhibit trypsin activity (100 µl) by about 60% when measured in the activity assay (see Section 6.1.8., supra). A 100 µl aliquot of the eluate was subjected to chromatographic separation by reverse phase HPLC on a Vydac C4 column (obtained from Western Analytical, Temecula, CA) in a linear gradient of 0 to 50% acetonitrile in 0.1% TFA over 12 minutes. The HPLC profile revealed one major peak of absorbance at 215 nm, and this peak was manually collected.

The HPLC A_{215} peak of material from runs 1040 and 1042 was evaluated in the activity assay (described in Section 6.1.8., supra). Two hundred microliters of the peak inhibited approximately 30% of the trypsin (100 µl) activity, while a sample of the HPLC effluent collected just after the peak showed no activity.

A 100 µl aliquot of the HPLC peak with trypsin inhibitory activity was loaded onto an Applied Biosystems gas phase protein sequencer, and the first ten amino acids of a protein sequence of KPI from runs 1040 and 1042 were determined. The determined sequence was (SEQ ID NO. 22):

E-V-V-R-E-V-X-S-E-Q,

which matches the expected sequence of nine of the first ten residues of KPI correctly processed from αMF prepro (see SEQ ID NO. 2). The X represents the one amino acid residue that could not be determined.

The amino acid sequence of the KPI analog produced by the SMD1189 strain was also determined. The first 24 amino acids of the KPI produced in run 1153 were determined, and confirm the mutation of Ile for Arg at amino acid residue 17 in the sequence (see SEQ. ID NO. 2).

8. EXAMPLE: PURIFICATION AND PROPERTIES OF KPI

8.1. MATERIALS AND METHODS

8.1.1. PURIFICATION OF KPI

Fermentor runs 1040, 1042, 1052, 1083 and 1153 were harvested and the cells were removed by centrifugation at~4000rpm, 10 min, 40°C. One liter of cell-free broth from runs 1040, 1042 and 1052 was diluted to four liters with 50 mM HOAc. One liter of cell broth from runs 1083 and 1153 was diluted with water and then mixed with 5 ml of concentrated sulfuric acid. The diluted broth from each run was loaded onto its own Kontes column (approximately 60 ml) packed with Spherodex cation exchange resin (IBF). The loading effluent from each column was collected in four one-liter aliquots and evaluated in the trypsin inhibition activity assay (see Section 6.1.9., supra). Trypsin inhibitory activity assays of the loading effluent samples collected from columns loaded with broth from runs 1040, 1042, 1052 and 1083 were negative.

The columns loaded with broth from runs 1040, 1042, 1052 and 1083 were washed and equilibrated with approximately 500 ml of 50 mM NaH_2PO_4, pH 4.2. Columns loaded with broth from run 1153 were equilibrated in 50 mM acetic acid. The KPI bound to the column from run 1040 was eluted in a 500 ml linear gradient from 50 mM NaH_2PO_4 to 2X PBS (phosphate buffered saline). The KPI bound to the column from

run 1042 was eluted in a 500 ml linear gradient from 50 mM NaH_2PO_4 to 1X PBS. The results from the two gradients were essentially equivalent. KPI bound to the column from runs 1052, 1083 and 1153, and was eluted in 50 mM ammonium acetate, pH 5.5. The A_{280} of the effluent was monitored with an ISCO on-line detector and the absorption peak of eluates from all runs were collected manually. Fractions from eluates of columns loaded with broth from runs 1040, 1042, 1052 and 1083 were assayed for trypsin inhibitory activity and protein concentration (BCA attached Assay, Pierce Chemicals, Rockford, IL). To acheive even greater purification of the recombinant KPI and remove any residual colored contaminants from the purified material, the KPI-containing eluate from the cation exchange chromatography column loaded with broth from runs 1040, 1042, 1052 and 1093 was pooled and applied to a column containing Toyopearl butyl 650M (TosoHaas, Philadelphia, PA) hydrophobic interaction chromatography (HIC) resin. Ammonium sulfate was added to the pooled eluate to a final a concentration of 12% and the pH was adjusted to 7. If necessary, the eluate was diluted with 12% ammonium sulfate to insure that the KPI concentration was not greater than 2 g/l.

The KPI was eluted from the HIC column in a linear gradient of 12 - 0% ammonium sulfate. The A_{280} of the effluent was monitored as described above, and the absorption peak eluate was collected.

To remove the ammonium sulfate from the purified KPI, the eluate form the HIC column was diluted 10-fold with water and the pH was adjusted to 2.8 with phosphoric acid. The diluted material was loaded onto a column packed with Spherodex cation exchange resin. The column was washed with 0.2 M

acetic acid followed by 2 volumes of 0.02 M acetic acid. The KPI was eluted in 50 mM ammonium acetate. The A_{280} of the effluent was monitored as described above and the absorption peak eluate was collected and lyophilized directly. Amino acid analysis of the purified KPI was conducted using standard procedures.

8.1.2. PROTEASE INHIBITORY ACTIVITY OF KPI

Inhibition equilibrium constants were measured by the following procedure. The active sites of trypsin and chymotrypsin were titrated by the method of Chase and Shaw (1969, Biochemistry 8:2212-2224) using the burst titrant *p*-nitophenyl guanidinobenzoate. These proteases were then used to titrate KPI. Protein concentration of factor XIa was determined by the method of Smith et al. (1985, Anal. Biochem. 150:76-85) and specific activity was determined by amidolytic and coagulation assays as described (Walsh et al., 1984, J. Clin. Invest. 73:1392-1399). KPI complex formation as observed in titration experiments revealed 1:1 stoichiometry for trypsin. Measurements of the inhibition equilibrium contains (K_i) were based on the following interactions:

$$\begin{array}{ccccc} & & \overset{K_m}{} & & \\ E & + & S \rightleftharpoons ES & \rightarrow & E + P \\ + & & & & \\ I & & & & \\ K_i \updownarrow & & & & \\ EI & & & & \end{array} \quad \text{(Scheme 1)}$$

where *E*, S, P, and I represent protease, substrate, product, and inhibitor, respectively; K_m is the Michaelis constant for the protease-substrate reaction and K_i is the equilibrium constant for the protease-inhibitor reaction. K_i was determined by the method of Henderson (1972, Biochem J. 127:321-333) as simplified

by Bieth (1980, Clin. Respir. Physiol. 16:183-195). Briefly, increasing amounts of titrated KPI were incubated with a constant amount of protease in a volume of 100 µl of 500 mM Tris-HCl, 150mM NaCl, pH 7.5, containing 0.1% bovine serum albumin in triplicate in 96-well microtiter plates. Incubations were conducted at 25°C for 10 to 15 min. Remaining protease activity was then measured by adding 50 µl of an excess concentration of the corresponding chromogenic substrate and following the change in absorbance at 410 nm in a microtitre plate reader (Flow Laboratories). The rate of the reaction was followed for a sufficient time (usually 20-30 min) to obtain a steady-state rate of substrate hydrolysis. Graphical analysis yielded an apparent K_i, using the following equation:

$$[I]/(1 - a) = K_{i,app}(1/a) + [E] \quad \text{(Scheme 2)}$$

where [I] and [E] represent the initial concentrations of inhibitor and protease, respectively, and *a* is the remaining fractional protease activity. Since the inhibition is reversible, as shown in Scheme 1, a correction must be made for the K_m of the protease-substrate reaction. The K_m for each protease and substrate was determined independently and the true K_i was calculated using the following equation:

$$K_i = \frac{K_{iapp}}{1 + [S]/K_m}$$

8.1.3. BINDING ACTIVITY OF KPI

An aliquot of purified KPI was incubated with ^{125}I-labeled epidermal growth factor binding protein (EGF BP) (20-590 ng/ml) for 30 min at 25°C. [The mixture was run on SDS-page according to Laemnli

(1970, Nature). The gels were dried and exposed to X-ray film for 12-24 h at -70°C.

8.2. RESULTS

8.2.1. HIGHLY PURIFIED KPI

The results of the activity assay and Pierce BCA protein assay of KPI purified from broth of runs 1040 and 1042 indicated that 600 to 700 mg of KPI were recovered from each of these one liter runs. A typical trypsin inhibition assay with purified KPI and broth containing KPI, along with an aprotinin analog positive control, is shown in Figure 2. Measurement of trypsin inhibition activity relative to the purified aprotinin analog standard indicated that the BCA assay seemed to overestimate KPI concentration by a factor of two.

KPI of high purity crystallized during cold (4° C) storage of the KPI-containing eluate fractions from the first Spherodex-based chromatography step in purification of KPI from broth of runs 1040 and 1042. The purity was assayed at greater than 98% by HPLC.

The KPI concentrations of the preparations purified from the broth of 10-liter fermentations of strains G+KPI200S3 and G+KPI200S20, runs 1052 and 1083, respectively were approximately 3.5 and 8.3 g, respectively. Amino acid analysis of the KPI purified from broth of strain G+KPI200S3 (run 1062) using cation exchange and hydrophobic interaction chromatography was consistent with the expected amino acid composition, as shown in Table V.

TABLE V.

KPI AMINO ACID COMPOSITION
BY AMINO ACID ANALYSIS

		Found			
		Experiment 1		Experiment 2	
AA	Expected	Mm	Residues	nM	Residues
D	5	43	5.4	34.5	5.1
T	3	20	2.5	18	2.6
S	3	22	2.8	21	3.0
E	8	92	11.8	68	9.9
P	2	ND	--	ND	--
G	7	54	6.8	51	7.5
A	5	45	5.7	41	6.0
C	6	39	4.9	36	5.2
V	5	36	4.6	34	4.9
M	2	13	1.6	13	1.9
I	2	16	2.0	16	2.3
L	0	--	--	--	--
Y	3	24	3.0	14.5	2.1
F	4	33	4.2	25	3.6
H	0	--	--	--	--
K	1	8	1.0	7.7	1.1
R	4	33	4.2	30	4.3
W	1	ND	--	ND	--

ND = Not Determined

8.2.2. KPI INHIBITS PROTEASE ACTIVITY SIMILARLY TO PN-2/APP

Protease inhibitory activity of PN-2 (soluble APP containing the KPI, obtained according to

Van Nostrand et al., 1990, J. Biol. Chem. 265:9591-9594)and expressed KPI were compared. The results of this comparison are shown in Table VI.

TABLE VI

PROTEASE INHIBITORY ACTIVITY OF PN-2 AND KPI

	Ki (M)[2]	
Protease	PN-2/APP	KPI
Factor Xla + 10 U/ml heparin[1]	$5.5 \pm 3.5 \times 10^{-11}$	$3.1 \pm 0.6 \times 10^{-10}$
Factor Xla	$4.0 \pm 0.8 \times 10^{-10}$	$4.5 \pm 1.8 \times 10^{-10}$
Trypsin	$8.3 \pm 2.2 \times 10^{-10}$	$3.1 \pm 1.2 \times 10^{-10}$
Chymotrypsin	$3.8 \pm 1.2 \times 10^{-9}$	$2.3 \pm 0.9 \times 10^{-9}$

[1] The substrate and its final molarity were Pro-Glu-Pro-Arg-p-nitroanilide HCl, 0.5 mM (Factor XIa); carbobenzoxy-Val-Gly-Arg-4-nitroanilide, 0.5 mM (trypsin); and N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, 0.25 mM (chymotrypsin).

[2] Ki is the equilibrium constant for the protease-inhibitor reaction, which was determined as described in Van Nostrand et al., 1990, J. Biol. Chem. 265:9591-9594.

The sixty one amino acid KPI-containing fragment expressed in this invention displayed similar protease inhibitory properties to that of Protease Nexin-2. However, from the data presented in Table VI, it is clear that the sixty one amino acid KPI-containing fragment was not capable of having its inactivation of factor XIa augmented by heparin. To the contrary, heparin is capable of significantly accelerating the inactivation of factor XIa by PN-2. This strongly suggests that heparin binds to regions on PN-2 which are not within the KPI domain.

PN-2 has been shown to be capable of forming SDS-stable complexes with 125I-labeled proteases such as trypsin and EGFbp (Van Nostrand and Cunningham, 1987, J. Biol. Chem. 262:8508-8514). The KPI-

containing sixty one amino acid fragment was not capable of forming SDS-stable complexes with either of these radioiodinated serine proteases suggesting that additional domains present on PN-2 but not the sixty one amino acid KIP-containing fragment are responsible for this activity.

A titration of KPI against 10 nM trypsin showed quantitative inhibition of trypsin activity by KPI in a 1:1 molar ratio (Figure 3). These results are consistent with the expected mechanism of Kunitz-type protease inhibitors, which stoichiometrically inhibit proteases.

A protease, such as epidermal growth factor binding protein (EGF BP) binding assay with KPI was negative in the presence of SDS (data not shown). These results stand in contrast to PN-2, which was shown to form SDS-stable complexes with EGF BP, nerve grow factor (NGF)-γ, and trypsin (Van Nostrand et al., 1990, supra).

9. IN VITRO MODEL FOR APP PROCESSING

An approach has been developed using an *in vitro* model system for APP processing and amyloidogenesis. KPI, which is an agent that at relatively high concentrations ($\geq$ 10mM), appears capable of altering the processing of APP by neuronal cells, is added to *in vitro* cultures of neuronal-type cells and the processing of APP is monitored in the conditioned medium.

Neuronal-type cell lines have been identified which secrete high levels of APP. APP processing can be monitored by analyzing aliquots of the conditioned medium from these cells. We have identified KPI (at high concentrations) as an agent

that appears to alter the carboxyl terminal processing of APP by these neuronal cell types.

9.1. MATERIALS AND METHODS

Human neuroblastoma SHSY5Y cells were grown in T-75 tissue culture flasks to 60-80% confluency in tissue culture medium (DMEM supplemented with 10% fetal calf serum, antibiotics and L-glutamine). T-75 flasks containing equal numbers of viable cells were treated with various concentrations of the Kunitz protease inhibitory (KPI) domain of the APP. The KPI was prepared according to the method described in Sections 6 and 8, supra. SHSY5Y cells were treated with concentrations of KPI ranging from 1 μM to 1 mM over 72-hour period. Aliquots of the conditioned medium were taken at 0 hours, 12 hours, 24 hours, 48 hours and 72 hours post treatment. Aliquots of the conditioned medium were collected at the various time points and dialyzed against 20 volumes of phosphate-buffered saline (PBS) and then concentrated by purification over DEAE sepharose as described by Van Nostrand et al. (1990, J Biol. Chem. 265:9591-9594). Immunoblots were then performed on the concentrated conditioned medium using the anti-PN-2 monoclonal antibody mAbP2-1 (International Patent Publication WO 91/16628) by Van Nostrand et al., incorporated herein by reference in its entirety) as the probe. This antibody is reactive with an N-terminal epitope on APP (and PN-2).

9.2. RESULTS AND DISCUSSION

Secreted APP can be detected in the conditioned medium of SHSY5Y neuroblastoma cells (Figure 4). In the untreated cultures, conditioned medium contains APP which migrates primarily as one

band at approximately 110 kD. This is presumably the secretase product (i.e., PN-2). Secretase is an as yet undefined protease which cleaves within the middle of the β-protein domain of APP inserted in the plasma cell membrane. The cleavage that it performs occurs after lysine residue number 16 of the β-protein. The secretase is most likely an extracellular serine protease based on its substrates' location and specificity. The produce of the secretase cleavage is PN-2 (when APP contains the KPI domain).

When the SHSY5Y cells were treated with relatively high concentrations of KPI (10 μM to 1 mM), C-terminal truncated forms of AβPP appear in the conditioned medium of these cells. This effect was most robust when KPI concentrations of 100 μM to 1 mM were used. This suggests that at relatively high concentrations KPI is influencing the procesing of APP. Other serine protease inhibitors such as aprotinin and chymostatin can be tested in this assay as well. Cell viability assays using trypan blue exclusion suggests that this C-terminal truncated product was not simply due to cell death.

Although the data are not definitive, the molecular weight of the C-terminal truncated fragment is consistent with processing of APP to form β-amyloid protein. The presence of β-amyloid is inferred from the size of the APP product detected with the monoclonal antibody.

The present invention is not to be limited in scope by the specific embodiments described herein since such embodiments are intended as but single illustrations of one aspect of the invention and any microorganisms which are functionally equivalent are within the scope of this invention. Indeed, various

modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for the purpose of description.

Various references are cited herein, the disclosures of which are incorporated by reference herein in their entirety.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Salk Institute Biotechnology/Industrial Associates and Wagner, Steven, L. et al.

(ii) TITLE OF INVENTION: High Level Expression Of The Amyloid Precursor Protein Protease Inhibitor Domain And Treatments Of Amyloidosis, Coagulation Disorders and Head Trauma Based Thereon

(iii) NUMBER OF SEQUENCES: 25

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pennie & Edmonds
(B) STREET: 1155 Avenue of the Americas
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) ZIP: 10036-2711

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 30-OCT-1992
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Misrock, S. Leslie
(B) REGISTRATION NUMBER: 18,872
(C) REFERENCE/DOCKET NUMBER: 7416-005-228

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212 790-9090
(B) TELEFAX: 212 869-8864/9741
(C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 204 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 7..190

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
AAGCTT GAG GTT GTT AGA GAG GTT TGT TCT GAG CAA GCT GAG ACT GGT          48
       Glu Val Val Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly
         1               5                  10

CCA TGT AGA GCT ATG ATT TCT AGA TGG TAC TTC GAC GTT ACT GAG GGT          96
Pro Cys Arg Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly
 15                  20                  25                  30
```

```
AAG TGT GCT CCA TTC TTC TAC GGT GGT TGT GGT GGT AAC AGA AAC AAC        144
Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn
                35                  40                  45

TTC GAC ACT GAG GAG TAC TGT ATG GCT GTT TGT GGT TCT GCT ATT T          190
Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
            50                  55                  60

AAGAATTCGG ATCC                                                        204
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Glu Val Val Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys
 1               5                  10                  15

Arg Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys
            20                  25                  30

Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp
        35                  40                  45

Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 453 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
GAATTCATGA GATTTCCTTC AATTTTTACT GCAGTTTTAT TCGCAGCATC CTCCGCATTA      60

GCTGCTCCAG TCAACACTAC AACAGAAGAT GAAACGGCAC AAATTCCGGC TGAAGCTGTC     120

ATCGGTTACT CAGATTTAGA AGGGGATTTC GATGTTGCTG TTTTGCCATT TTCCAACAGC     180

ACAAATAACG GGTTATTGTT TATAAATACT ACTATTGCCA GCATTGCTGC TAAAGAAGAA     240

GGGGTATCTT TGGATAAAAG AGAGGTTGTT AGAGAGGTTT GTTCTGAGCA AGCTGAGACT     300

GGTCCATGTA GAGCTATGAT TTCTAGATGG TACTTCGACG TTACTGAGGG TAAGTGTGCT     360

CCATTCTTCT ACGGTGGTTG TGGTGGTAAC AGAAACAACT TCGACACTGA GGAGTACTGT     420

ATGGCTGTTT GTGGTTCTGC TATTTAAGAA TTC                                  453
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGCTTGAGGT TGTTAGAGAG GTTTGTTCTG AGCAAGCTGA GACTG 45

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTCCATGTAG AGCTATGATT TCTAGATGGT ACTTCGACGT TACTGAGGGT 50

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGTGTGCTC CATTCTTCTA CGGTGGTTGT GGTGGTAACA GAAACAACTT 50

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGACACTGAG GAGTACTGTA TGGCTGTTTG TGGTTCTGCT ATTTAAGAAT TCG 53

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACATACCGAC AAACACCAAG ACGATAAATT CTTAAGCCTA G 41

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGATGCCACC AACACCACCA TTGTCTTTGT TGAAGCTGTG ACTCCTCATG 50

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTAAAGATCT ACCATGAAGC TGCAATGACT CCCATTCACA CGAGGTAAGA 50

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTCCAACAA TCTCTCCAAA CAAGACTCGT TCGACTCTGA CCAGGTACAT CTCGATA 57

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AATTCGATGA GATTTCCTTC AATTTTTACT GCA 33

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTAAAAATTG AAGGAAATCT CATCG 25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGGGTATCTT TGGATAAAAG AGAGGTTGTT AGAGAGGTTT GT 42

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GATAAAAGAG AGGTTGTT 18

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TACTTCGACG TTACTGAGGG TAAGTGTGCT CCATTCT 37

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTTACTGAGG GTAAGTGT 18

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAATTAATTC 10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GAATTAATTC 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAGCAGATCT GCTG 14

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

14

CAGCAGATCT GCTG

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Glu Val Val Arg Glu Val Xaa Ser Glu Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTGAGACTGG TCCCATGTGT CGCGATGATT TCTAGATGGT ACT 43

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GATTAGAATC TAGCAAGACC 20

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CATAATTGCG ACTGGTTCC 19

WHAT IS CLAIMED IS:

1. A recombinant DNA molecule encoding a Kunitz-type protease inhibitor comprising the nucleotide coding sequence substantially as depicted in SEQ ID NO. 3.

2. The recombinant DNA molecule of Claim 1 operationally associated with a regulatory nucleotide sequence that controls expression of the coding sequence in a host cell.

3. The recombinant DNA molecule of Claim 2 in which the nucleotide coding sequence further encodes a secretory signal that provides for secretion of the expressed product by a host cell.

4. A recombinant DNA molecule comprising a nucleotide sequence encoding a Kunitz-type protease inhibitor having the amino acid sequence depicted in SEQ ID NO. 2, operationally associated with a regulatory sequence that controls expression of the Kunitz-type protease inhibitor and a secretory signal that provides for secretion of the Kuntz-type protease inhibitor.

5. A recombinant DNA molecule, pKPI200.

6. A recombinant DNA molecule, pKPIAV.21.

7. A cultured cell which contains the recombinant DNA molecule of Claim 1.

8. A cultured cell which contains the recombinant DNA molecule of Claim 2 and which expresses the Kunitz-type protease inhibitor.

9. A cultured yeast cell which contains the recombinant DNA molecule of Claim 2 and which expresses the Kunitz-type protease inhibitor.

10. The cultured yeast cell of Claim 8 in which the regulatory sequence is the AOX1 promoter.

11. The cultured yeast cell of Claim 8 in which the yeast cell is a methylotrophic yeast.

12. The cultured yeast cell of Claim 11 in which the yeast cell species is Pichia pastoris.

13. The cultured Pichia pastoris of Claim 12 in which the strain is G+KPI200S20, G+KPI200S2, G+KPI200S3, or G+KPI200S4.

14. The cultured Pichia pastoris of Claim 12 in which the strain is SMD1189.

15. A cultured cell which contains the recombinant DNA molecule of Claim 3 and which expresses and secretes the Kunitz-type protease inhibitor.

16. A cultured yeast cell which contains the recombinant DNA molecule of Claim 3 and which expresses and secretes the Kunitz-type protease inhibitor.

17. The cultured yeast cell of Claim 16 in which the secretory signal is the pre-pro signal sequence for α-mating factor.

18. A yeast cell transformed with pKPI200.

19. A yeast cell transformed with pKPIAV.21.

20. The yeast cell of claim 18 or 19 which is a methylotrophic yeast.

21. The methylotrophic yeast cell of claim 20 which is a Pichia pastoris.

22. Pichia pastoris of Claim 21 in which the strain is G+KPI200S20, G+KPI200S2, G+KPI200S3, G+KPI200S4 or SMD1189.

23. A cultured yeast cell which contains a recombinant DNA molecule comprising a nucleotide sequence encoding a Kunitz-type protease inhibitor having the amino acid sequence depicted in SEQ ID NO. 2, operationally associated with a regulatory sequence that controls expression of the Kunitz-type protease inhibitor by the cultured cell.

24. The cultured yeast cell of Claim 23 in which the nucleotide coding sequence further encodes a secretory signal that provides for secretion of the expressed product by the cultured yeast cell.

25. The cultured yeast cell of Claim 23 in which the regulatory sequence is the AOX1 promoter.

26. The cultured yeast cell of Claim 24 in which the secretory signal is the pre-pro signal sequence for α-mating factor.

27. The cultured yeast cell of Claim 23 which is a methylotrophic yeast.

28. The cultured yeast cell of Claim 27 which is Pichia pastoris.

29. The cultured yeast cell of Claim 28 in which the strain of Pichia pastoris is G+KPI200S20, G+KPI200S2, G+KPI200S3, or G+KPI200S4.

30. A method for producing a Kunitz-type protease inhibitor, comprising:
    (a) culturing a host cell transformed with a recombinant DNA molecule comprising the nucleotide coding sequence substantially depicted in SEQ ID NO. 3, operationally associated with a regulatory sequence that controls expression of the coding sequence, so that a Kunitz-type protease inhibitor is expressed by the cultured cell; and
    (b) recovering the Kunitz-type protease inhibitor so expressed from the cell culture.

31. The method of Claim 30 in which the cultured host cell is a yeast cell.

32. The method of Claim 31 in which the regulatory sequence is the AOX1 promoter.

33. The method of Claim 31 in which the yeast cell species is Pichia pastoris.

34. The method of Claim 33 in which the strain of Pichia pastoris is G+KPI200S20, G+KPI200S2, G+KPI200S3, or G+KPI200S4.

35. The method of Claim 33 in which the strain of Pichia pastoris is SMD1189.

36. The method of Claim 30 in which the nucleotide coding sequence further encodes a secretory signal that provides for secretion of the expressed product by the cultured cell, so that the Kunitz-type protease inhibitor is recovered from the culture media.

37. The method of Claim 36 in which the cultured host cell is a yeast cell.

38. The method of Claim 37 in which the secretory signal is the pre-pro signal sequence of α-mating factor.

39. The method of Claim 37 in which the yeast cell is a methylotrophic yeast.

40. The method of Claim 39 in which the yeast cell species is Pichia pastoris.

41. The method of Claim 40 in which the strain of Pichia pastoris is G+KPI200S20, G+KPI200S2, G+KPI200S3, or G+KPI200S4.

42. The method of Claim 40 in which the strain of Pichia pastoris is SMD1189.

43. A method for producing a Kunitz-type protease inhibitor, comprising:

(a) culturing a yeast cell transformed with a recombinant DNA molecule encoding a Kunitz-type protease inhibitor having the amino acid sequence depicted in SEQ ID NO. 2, operationally associated with a regulatory sequence that controls expression of the coding sequence so that a Kunitz-type protease inhibitor is expressed by the cultured yeast cell; and

(b) recovering the Kunitz-type protease inhibitor so expressed from the culture.

44. The method of Claim 43 in which the coding sequence further encodes a secretory signal that provides for secretion of the expressed product by the cultured yeast cell.

45. The method of Claim 43 in which the regulatory sequence is the AOX1 promoter.

46. The method of Claim 44 in which the secretory signal is the pre-pro signal sequence for α-mating factor.

47. The method of Claim 43 in which the cultured yeast cell is a methylotrophic yeast.

48. The method of Claim 47 in which the cultured yeast cell is Pichia pastoris.

49. The method of Claim 48 in which the strain of Pichia pastoris is G+KPI200S20, G+KPI200S2, G+KPI200S3, or G+KPI200S4.

50. A method for producing a Kunitz-type protease inhibitor, comprising:
    (a) culturing Pichia pastoris transformed with recombinant plasmid pKPI200, so that a Kunitz-type protease inhibitor is expressed by the cultured cell; and
    (b) recovering the Kunitz-type protease inhibitor so expressed from the culture.

51. The method according to Claim 44 in which the strain of Pichia pastoris is G+KPI200S20, G+KPI200S2, G+KPI200S3, or G+KPI200S4.

52. A method for producing a Kunitz-type protease inhibitor, comprising:
    (a) culturing Pichia pastoris transformed with recombinant plasmid pKPIAV.21, so that a Kunitz-type protease inhibitor is expressed by the cultured cell; and
    (b) recovering the Kunitz-type protease inhibitor so expressed from the culture.

53. The method according to Claim 44 in which the strain of Pichia pastoris is SMD1189.

54. A method for treating amyloidosis in a subject comprising administering an effective dose of a Kunitz-type protease inhibitor to the subject.

55. The method of Claim 54 in which the subject has Alzheimer's Disease, Down's syndrome, neuronal ceroid lipofuscinosis, cerebral amyloid angiopathy, hereditary cerebral hemorrhage with amyloidosis - Dutch and Icelandic or dementia pugilistica.

56. A method for treating a coagulation disorder in a subject comprising administering an effective dose of a Kunitz-type protease inhibitor to the subject.

57. A method for inhibiting the protease activity of serine proteases in a subject comprising administering an effective dose of a Kunitz-type protease inhibitor to the subject.

58. A method for inhibiting Factor XIa in a subject comprising administering an effective dose of a Kunitz-type protease inhibitor to the subject.

59. A method for treating head trauma in a subject comprising administering an effective dose of a Kunitz-type protease inhibitor to the subject.

60. The method of Claim 54, 56, 47, 58 or 59 in which the Kunitz-type protease inhibitor has the amino acid sequence depicted in SEQ ID NO. 2.

61. A method for treating emphysema, cystic fibrosis, and acute pancreatitis in a subject comprising

administering an effective amount of a Kunitz-type protease inhibitor to the subject.

62. A pharmaceutical composition in which the active ingredient comprises a Kunitz-type protease inhibitor produced by the method of Claim 30, 35 or 43.

63. A pharmaceutical composition in which the active ingredient comprises a Kunitz-type protease inhibitor produced by the method of Claim 50 or 52.

64. An *in vitro* method for elucidating the role of a Kunitz-type protease inhibitor in amyloid precursor protein processing comprising

(a) treating neuronal-type cells growing in a culture medium with a Kunitz-type protease inhibitor;

(b) detecting processed amyloid precursor protein in the culture medium;

(c) detecting processed amyloid precursor protein in culture medium of the neuronal-type cells that were not treated with the Kunitz-type protease inhibitor; and

(d) comparing the processed amyloid precursor protein detected in the culture medium of the treated cells with the processed amyloid precursor protein detected in the culture medium of the untreated cells

in which a difference in the processed amyloid precursor proteins compared in step (d) indicates

a role of the Kunitz-type protease inhibitor in amyloid precursor protein processing.

65. The method of Claim 64 in which the neuronal-type cells are neuroblastoma cells.

66. The method of Claim 56 in which the neuroblastoma cells are SHSY5Y cells.

67. The method of claim 64 in which the Kunitz-type protease inhibitor is produced according to the method of claim 26, 31 or 37.

68. The method of claim 64 in which the Kunitz-type protease inhibitor is produced according to the method of claim 50.

69. The method of claim 64 in which the Kunitz-type protease inhibitor has the amino acid sequence depicted in SEQ ID NO. 2.

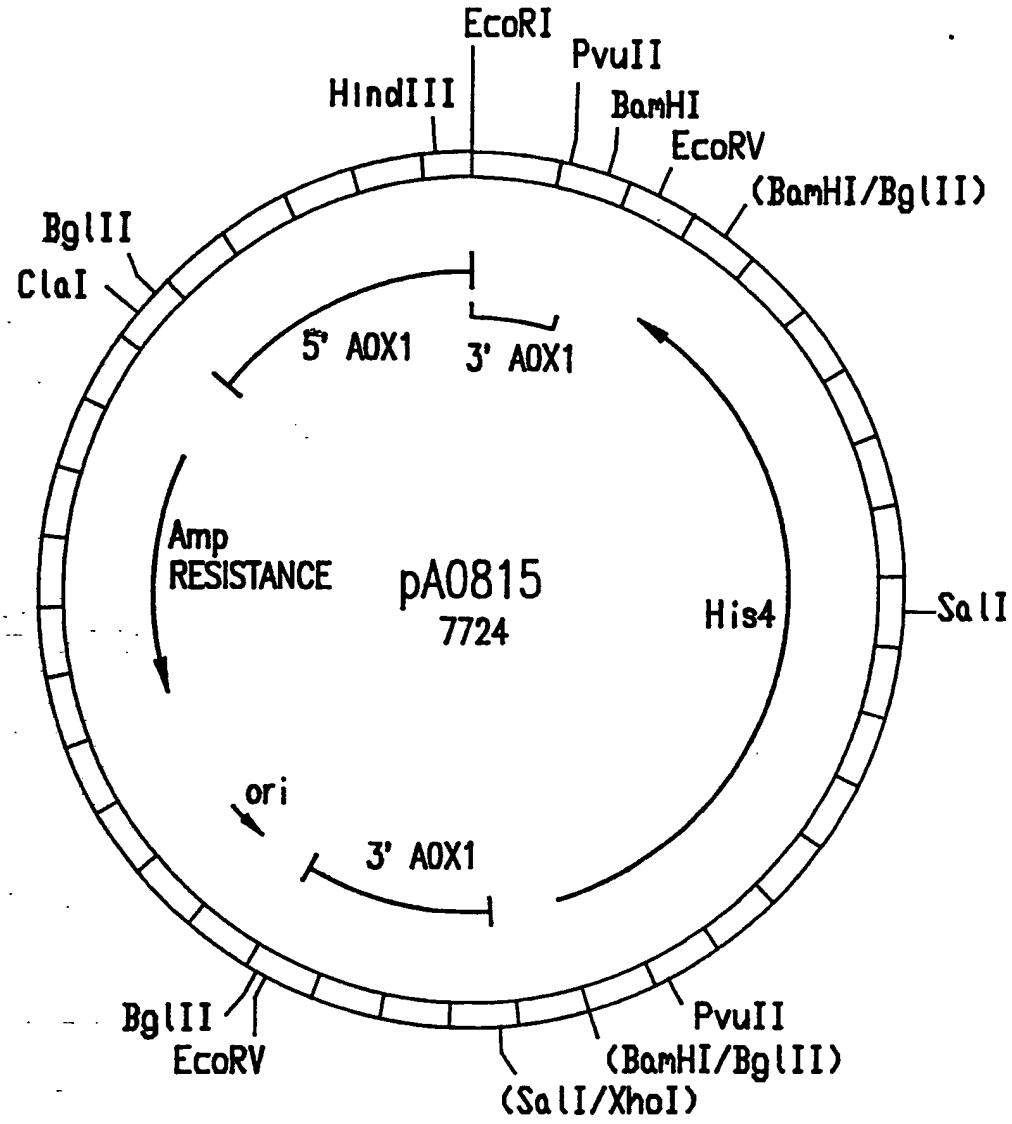

EcoRI
PvuII
HindIII
BamHI
EcoRV
(BamHI/BglII)
BglII
ClaI
5' AOX1
3' AOX1
Amp
RESISTANCE
pA0815
7724
His4
SalI
ori
3' AOX1
BglII
EcoRV
PvuII
(BamHI/BglII)
(SalI/XhoI)

FIG.1A

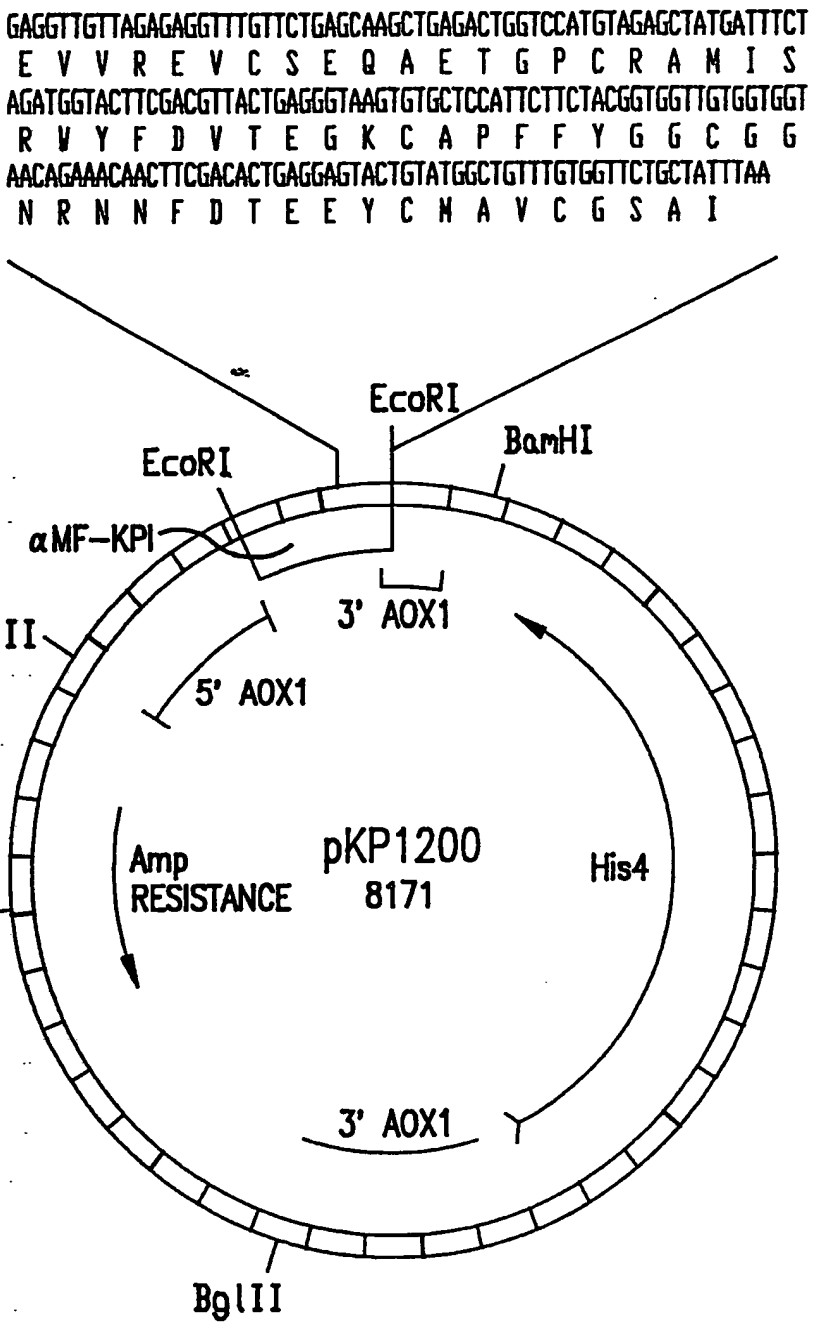

GAGGTTGTTAGAGAGGTTTGTTCTGAGCAAGCTGAGACTGGTCCATGTAGAGCTATGATTTCT
E V V R E V C S E Q A E T G P C R A M I S
AGATGGTACTTCGACGTTACTGAGGGTAAGTGTGCTCCATTCTTCTACGGTGGTTGTGGTGGT
R W Y F D V T E G K C A P F F Y G G C G G
AACAGAAACAACTTCGACACTGAGGAGTACTGTATGGCTGTTTGTGGTTCTGCTATTTAA
N R N N F D T E E Y C M A V C G S A I
EcoRI
EcoRI
BamHI
αMF-KPI
3' AOX1
BglII
5' AOX1
Amp
RESISTANCE
pKP1200
8171
His4
PstI
3' AOX1
BglII

FIG.1B

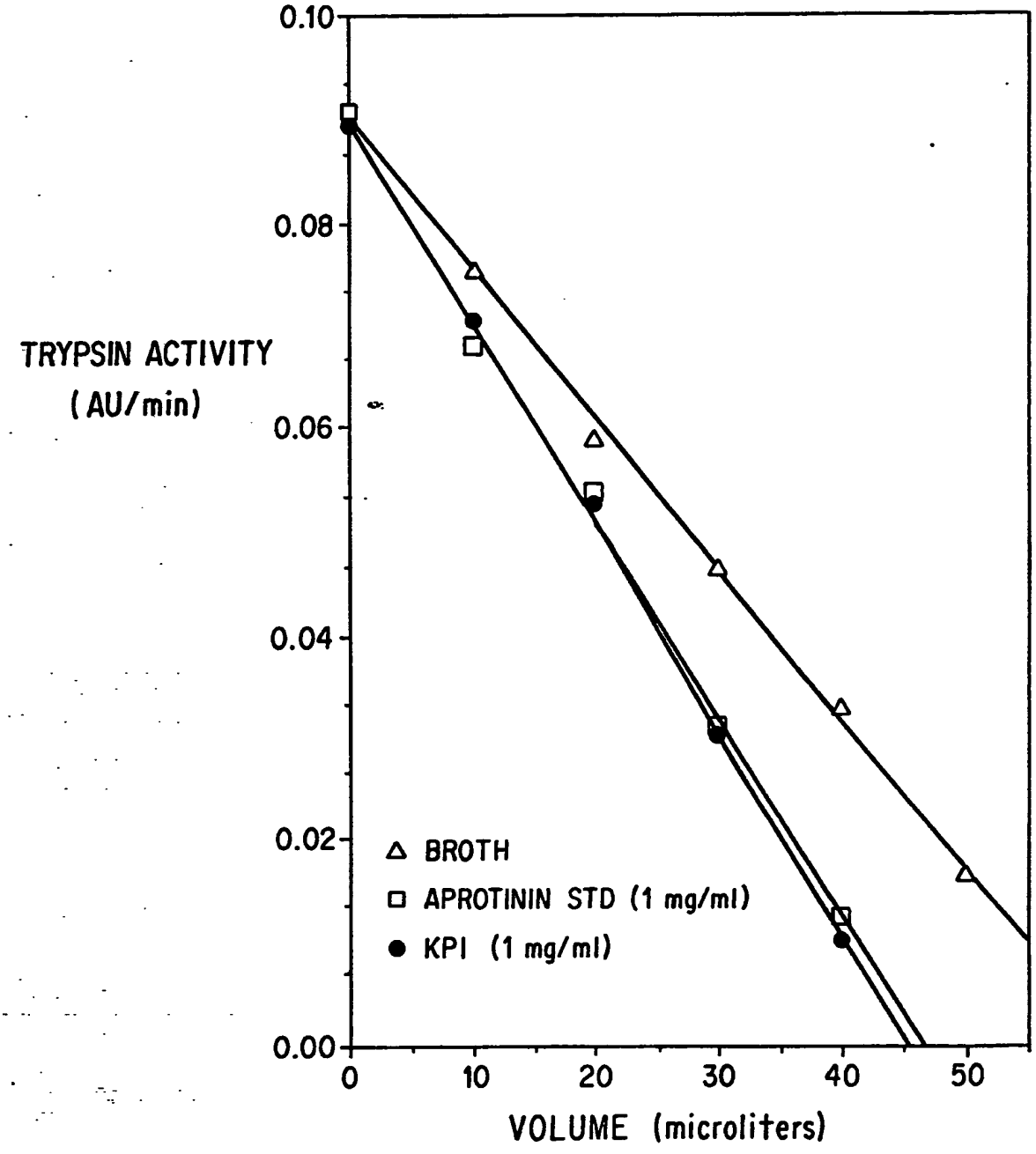

TRYPSIN ACTIVITY
(AU/min)
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
VOLUME (microliters)
△ BROTH
□ APROTININ STD (1 mg/ml)
● KPI (1 mg/ml)

FIG. 2

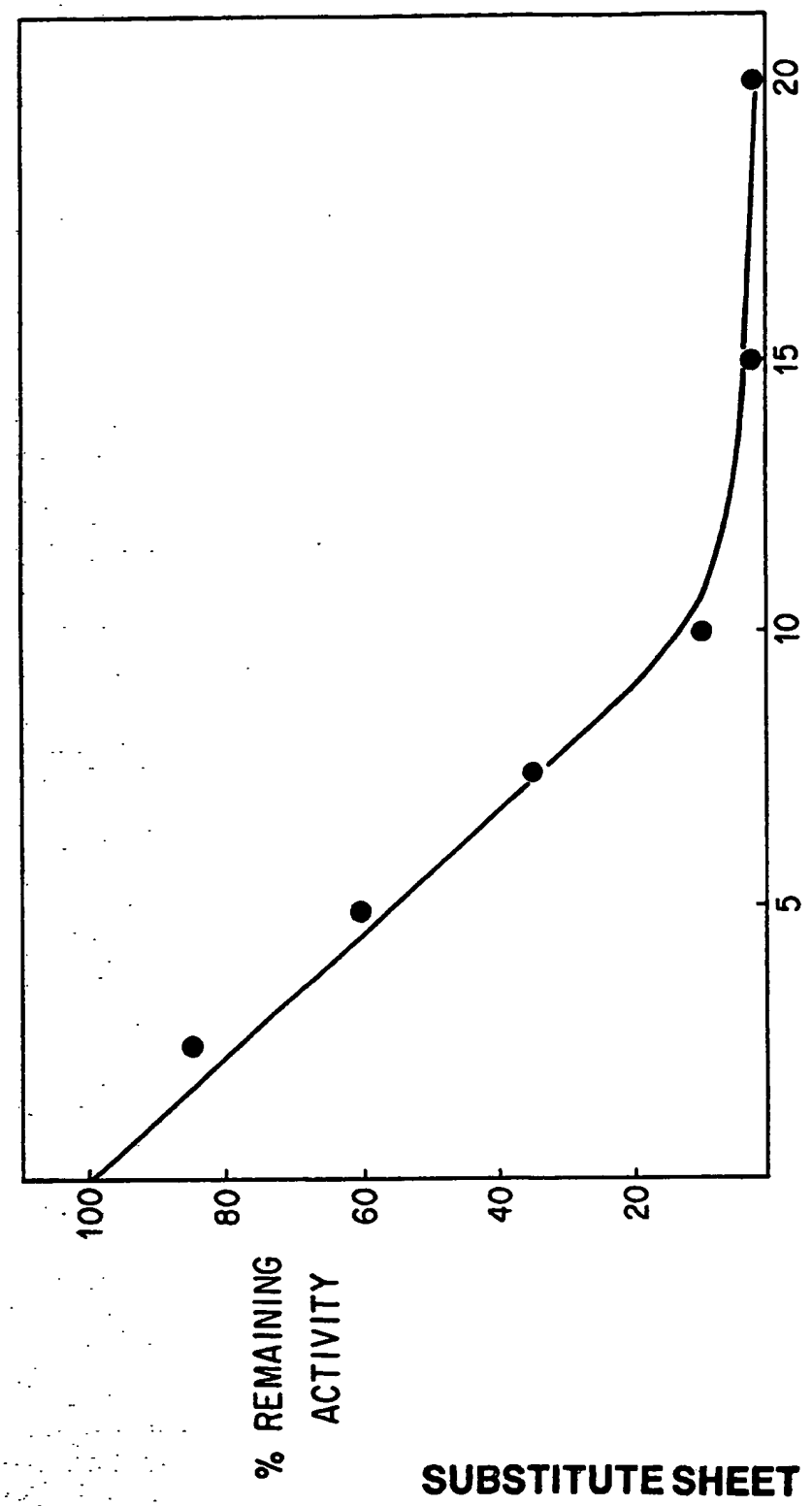


FIG. 3

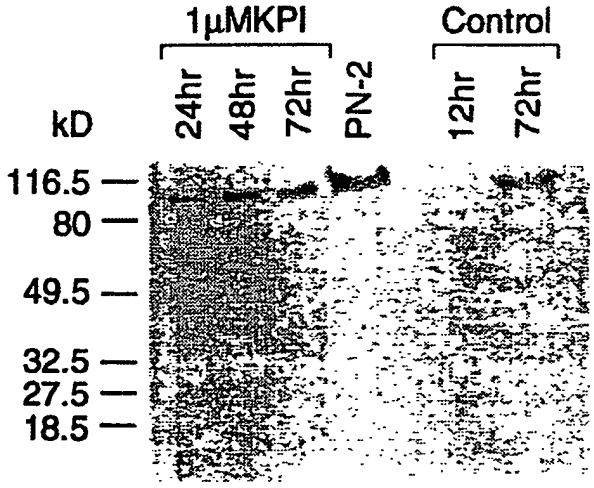

1μMKPI
Control
kD
24hr
48hr
72hr
PN-2
12hr
72hr
116.5
80
49.5
32.5
27.5
18.5

FIG. 4A

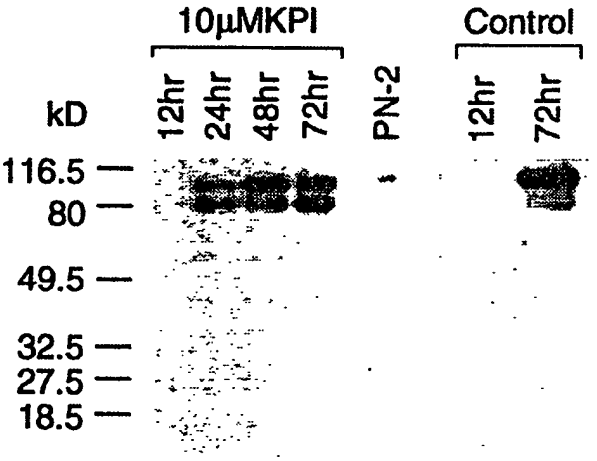

10μMKPI
Control
kD
12hr
24hr
48hr
72hr
PN-2
12hr
72hr
116.5
80
49.5
32.5
27.5
18.5

FIG. 4B

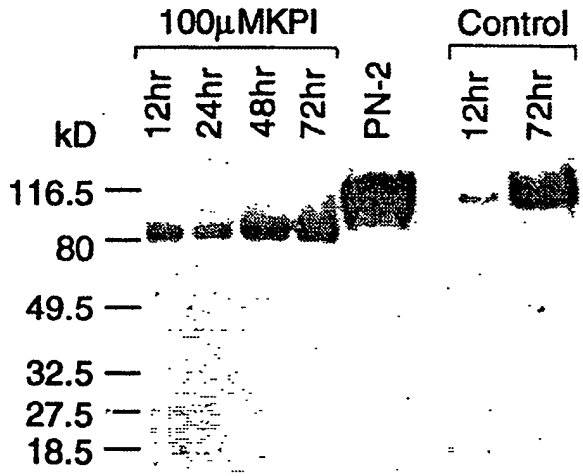

100μMKPI
Control
12hr
24hr
48hr
72hr
PN-2
12hr
72hr
kD
116.5
80
49.5
32.5
27.5
18.5

FIG. 4C